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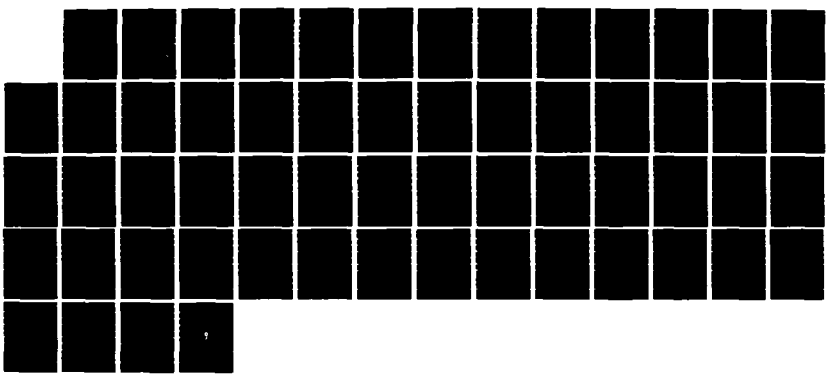

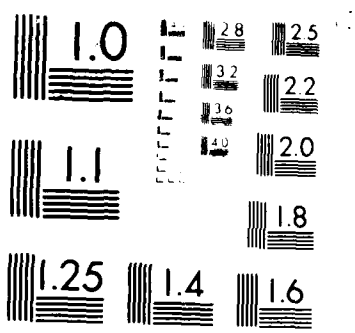

MICROCOPY RESOLUTION TEST CHART

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New Disinfection Agents For Water

ANNUAL REPORT

S. D. Worley, L. J. Swango, J. L. Aull, H. H. Kohl, D. E. Williams,
S. B. Barnela, U. Barnela, A. Killen, and L. Kong

MARCH 1985

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012
Project Officer James C. Eaton

and

HQ AFESC/RDVW
Tyndall Air Force Base, FL 32403
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Contract No. DAMD17-82-C-2257

Auburn University
Auburn University, Alabama 36849

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Further comparisons of 3-chloro-4,4-dimethyl-2-oxazolidinone (Compound I) with calcium hypochlorite (HTH) as field water disinfectants have been made. A mixture of 10 parts Compound I and 1 part HTH at low total chlorine concentration (1 mg/L) was able to kill S. aureus in demand-free water rapidly at time zero due to the small amount of HTH present and then with Compound I disinfection kinetics upon several rechallenges over a 1-month period. Compound I was considerably more stable than was HTH in demand-free water under [illegible] (25°C) except in highly alkaline water (pH 9.5). Seven N-halamine [illegible] synthesized during the past contract year. Most of these compounds were [illegible] than Compound I; one of the compounds was more stable in water than Compound I. The [illegible] of Compound I and HTH against rotavirus were compared under a [illegible] with HTH being the better disinfectant. In similar experiments [illegible] Giardia lamblia [illegible] was more effective than HTH in (over)

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19. Abstract

water disinfection. Mechanistic studies showed that Compound I attacks sulfhydryl groups on enzymes more rapidly than does HTH; on the other hand, aromatic amino acids react more efficiently with HTH than they do with Compound I. The mechanisms of action of the two compounds are clearly different and dependent upon the nature of the organism.

PREFACE

This report was prepard by the Water Resources Research Institute at Auburn University, Auburn, AL 36849 sponsored jointly by the U.S. Army Research and Development Command, Fort Detrick, Frederick, MD 21701 and the Air Force Engineering and Services Center, Air Force Engineering and Services Laboratory, Tyndall, FL 32403, under Contract Number DAMD17-82-C-2257.

This report covers work performed between September 1983 and November 1984. The Principal Investigator for the project was Dr. S. D. Worley of the Department of Chemistry at Auburn University. Project officers were Mr. James C. Eaton of USAMRDC, Fort Detrick, and Capt. Jack H. Jeter of HQ AFESC/RDVW, Tyndall AFB, FL.

This report discusses further comparison of a new N-chloramine compound with calcium hypochlorite (HTH) as a water disinfectant for military field use. Prior work and research protocols were discussed in detail in AFESC report ESL-TR-83-68 which was released in October 1984. This report also discusses the synthesis and preliminary testing of several new N-halamine compounds as potential water disinfectants for military field use. Citations of commercial organizations and trade names in this report do not constitute an official Department of Defense endorsement, approval, or rejection of the products or services of these organizations.

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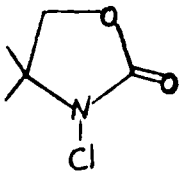
SECTION I

INTRODUCTION

The agent currently employed by the U.S. Army and U.S. Air Force for disinfection of field water supplies is HTH which is composed of 65 percent calcium hypochlorite and 35 percent "inert ingredients". While HTH is reasonably bactericidal, it is not particularly stable either in water solution or in the solid state. In fact improperly stored calcium hypochlorite can spontaneously ignite packaging materials (1). Furthermore, it is well known that "free chlorine" agents such as HTH react with organic impurities in water to produce toxic trihalomethanes, which have been shown to be carcinogenic in laboratory animals (2). It has been stated that N-chloramines do not react appreciably with organic material to produce toxic trihalomethanes (2). However, it has also been suggested that chloramines are much weaker bactericides than are free-chlorine agents (3). The research reported here is based upon the hypothesis that there are N-chloramine agents which are adequate disinfectants and which possess other attributes which render them of possible use to the military as all-purpose water disinfectants.

One such N-chloramine agent (3-chloro-4,4-dimethyl-2-oxazolidinone, henceforth referred to as Compound I) has been studied extensively in these laboratories. Compound I was first prepared



Compound I

and shown to be bactericidal by Kaminski and coworkers (4,5). Recent experiments in these laboratories have demonstrated that Compound I is an effective bactericide in a laboratory water treatment plant (6), that Compound I is exceptionally stable in water and in dry storage (7), that Compound I eradicates a broad spectrum of bacteria in water (8), that Compound I is apparently nontoxic to chickens drinking water containing the agent at the 200 ppm level (9), and that it detoxifies aflatoxin (9). The cellular mechanisms of action of Compound I in inhibiting bacterial DNA, RNA, and protein synthesis have been addressed in these laboratories in a preliminary manner also (10). A general summary of the chemical and biological properties of Compound I was presented at the Fourth International Conference on Water Chlorination (11). All of this preliminary work led us to the conclusion that Compound I, or some other chloramine with similar molecular structure, might be a better water disinfectant than HTH for military use.

The results presented in the first annual report (AFESC ESL-TR-83-68) (12) have been discussed in depth in several recent publications (13-18). The reader is referred to the first annual report and these recent publications (12-18) for detailed research protocol discussions and access to all data and

discussion for work during the first contract year. Any changes in protocols employed during the second contract year will be discussed in this report. For the second contract year data reduction, plotting, and statistical testing were performed using the Statistical Analyses System (SAS) (19) operating on the Auburn University IBM 3033 computer. Projected disinfection times were calculated using the General Linear Models (GLM) procedure of SAS after performing a log+1 transformation on the dependent variable of cell density (20). The SAS regression model was specified as log (cfu/mL+1) = time to calculate the regression equation. Options of the GLM procedure allow for testing of homogeneity of slopes of two or more regression lines by using an analysis of covariance (ANCOVA). Solution stability studies were also analyzed using the GLM procedure where the data were analyzed for zero order or first order decay by regressing either mg/L total halogen on time or log (mg/L halogen) on time. In some experiments the homogeneity of slopes test was used for comparison of stability curve slopes.

In this report all chloramine concentrations are reported in ppm (mg/L) total chlorine expressed as potential total Cl^+, although it should be realized that for most of the chloramines studied little "free chlorine" is formed in water solution. For the bromamines studied, total halogen concentration will be expressed in terms of the molar equivalent to the total chlorine concentration from compound I, ie. 10 ppm total chlorine concentration for Compound I is the molar halogen equivalent to 22.5 ppm total bromine concentration for the brominated analog of Compound I (Compound IB). For the chloramines studied having more than one chlorine atom per molecule, the total chlorine concentration will be the actual total chlorine titration value, ie. one mole of a compound having two N-Cl functional groups will yield two moles of total chlorine (Cl^+) relative to one mole Cl^+ for Compound I.

The goals for the second year of contract DAMD17-82-C-2257 were to test mixtures of Compound I and HTH against bacteria and to compare the stabilities of the two compounds at 37°C (Task 6), to synthesize new chloramine and bromamine disinfectants (Task 7), to compare the action of the new compounds with Compound I as to germicidal activity and stability (Task 8), to perform analogous testing on viruses and protozoa (Task 9), to test those compounds proving to be at least equal to Compound I as disinfectants in an extensive manner (Task 10), and to perform mechanistic studies of the action of Compound I (Task 11).

In this report each task will be addressed in turn with modifications in experimental protocol, results, and discussion being presented. This material will be followed by sections giving primary conclusions and finally our recommendations.

SECTION II

TASK 6 (ADDITIONAL TESTING OF COMPOUND I AND HTH)

The goal of Task 6 was to continue comparisons of Compound I and HTH so as to better evaluate the potential of Compound I as a field disinfectant. During the first contract year it was shown that in general HTH kills microorganisms more rapidly and at lower total chlorine concentration than does Compound I (12); notable exceptions to this general observation were the protozoa Giardia lamblia and Entamoeba invadens. However, it was also shown that Compound I was much more stable than HTH in demand free water or in a "worst case water" containing heavy organic demand (12). This was true at all pH values (4.5, 7.0, 9.5) and temperatures (22°, 4°C) studied (12). From these results we concluded that a good, all-purpose disinfectant for military field use might be composed of a mixture of HTH (for rapid disinfection) and Compound I (for long-term disinfection). This hypothesis was tested first under Task 6.

Three different mixtures of HTH and Compound I having total chlorine ratios of 1:1, 1:5, and 1:10, respectively, at two different total chlorine concentrations (1 ppm and 5 ppm) were dissolved in demand-free water at pH 7.0, 22°C and tested versus Staphylococcus aureus, with challenges made at time zero, 1 week, and 1 month. The concentration of microorganisms at each challenge was about 1×10^6 cfu/mL. Aliquots were withdrawn at T_o and after 1, 2, 5, 10, 120, and 300 minutes of contact between the organisms and various mixtures of the two disinfectants. These aliquots were immediately quenched with thiosulfate solution and plated. Due to the vast number of data points accumulated and the nature of the experiment, a subjective grading of cell density on the plates was used rather than the usual serial dilution microdrop technique. Samples were scored on a scale of 0 to 4, where 0 indicated no growth of the organisms after 48 hours of plate incubation at 37°C, and 4 indicated completely confluent growth under the same conditions, with 1, 2, and 3 indicating intermediate growth as scored on a subjective basis. After the initial "seeding" of tubes containing the disinfectant mixtures with organisms and removing the timed aliquots, the tubes were tightly capped and stored at 22°C until a rechallenge at 10^6 cfu/mL at 1 week and then again at 1 month.

The results of this series of experiments are shown in Table 1. It is clear from these experiments that mixtures containing even very small amounts of HTH are quite effective against S. aureus on the first day. For the 1 week and 1 month rechallenges Compound I disinfection kinetics (12) are followed, for the HTH has decomposed by that time. We believe that a Compound I: HTH ratio of [illegible] at even very low total chlorine concentration (1 ppm) could be an effective military field water disinfectant because the small amount of Compound I which is stable for long time periods should maintain disinfection indefinitely. A note of caution should be added here. These experiments were conducted at pH [illegible], 22° only; we must perform analogous experiments as a function of pH, temperature, and water quality at a later time.

TABLE I. ACTION OF MIXTURES OF COMPOUND I AND HTH AGAINST STAPHYLOCOCCUS AUREUS (pH 7, 22°C)

Mixture Exp.[a,b,c]	In To	In 1	In 2	In 5	In 10	In 120	In 300	Wk To	Wk 1	Wk 2	Wk 5	Wk 10	Wk 120	Wk 300	Mo To	Mo 2	Mo 5	Mo 10	Mo 120	Mo 300
1:1 Ratio																				
5 ppm Mixture	4	1	0	0	0	0	0	0	0	0	0	0	0	0	0	4	4	4	0	0
2.5 ppm I[d]	4	4	4	4	4	0	0	0	4	4	4	4	0	0	0	4	4	4	0	0
2.5 ppm HTH[d]	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	4	4	4	4
1 ppm Mixture	4	0	0	0	0	0	0	0	4	4	4	4	1	0	0	4	4	4	2	0
0.5 ppm I	4	4	4	4	4	2	0	0	4	4	4	4	2	0	0	4	4	4	2	0
0.5 ppm HTH	4	2	0	0	0	0	0	0	4	4	4	4	4	4	0	4	4	4	4	4
5:1 Ratio																				
5 ppm Mixture	4	0	0	0	0	0	0	0	4	3	3	2	0	0	0	4	4	4	0	0
4.1675 ppm I	4	4	4	4	3	0	0	0	4	4	3	1	0	0	0	4	4	4	0	0
0.835 ppm HTH	4	0	0	0	0	0	0	0	4	4	4	4	3	4	0	4	4	4	4	4
1ppm Mixture	4	0	0	0	0	0	0	0	4	4	4	4	1	0	0	4	4	4	1	0
0.8335 ppm I	4	4	4	4	4	1	0	0	4	4	4	4	1	0	0	4	4	4	2	0
0.1667 ppm HTH	4	0	0	0	0	0	0	0	4	4	4	4	4	4	0	4	4	4	4	4
10:1 Ratio																				
5 ppm Mixture	4	0	0	0	0	0	0	0	4	4	4	2	0	0	0	4	4	4	0	0
4.49 ppm I	4	4	4	4	3	0	0	0	4	4	4	1	0	0	0	4	4	4	0	0
0.45 ppm HTH	4	2	0	0	0	0	0	0	4	4	3	4	4	4	0	4	4	4	4	4
1 ppm Mixture	4	3	0	0	0	0	0	0	4	4	4	4	0	0	0	4	4	4	0	0
0.91 ppm I	4	4	4	4	4	0	0	0	4	4	4	4	0	0	0	4	4	4	0	0
0.09 ppm HTH	4	3	1	1	1	1	-	0	4	4	4	4	4	4	0	4	4	4	4	4
Control	4	4	4	4	4	4	4	1	4	4	4	4	4	4	0	4	4	4	4	4

a. Challenges were made at the first day (In), after 1 week, and after 1 month.
b. Contact time in minutes.
c. Subjective plate scoring was used with a range of 0 for no growth up to 4 for confluent growth.
d. Pure component (either Compound I or HTH)

Also under Task 6 the stabilities of Compound I and HTH in demand-free water at pH 4.5, 7.0, and 9.5 have been compared at elevated temperature (37°C). The protocol for these experiments was presented in detail in the previous annual report (12). The solutions were held at 37°C by storage in an incubator. The results of these experiments are summarized in Figure 1.

The stability curves in Figure 1 were subjected to linear regression analysis in an attempt to generate a regression equation to best describe the change in total Cl^+ concentration with respect to time. Using the SAS procedure of GLM, an analysis of covariance to test for homogeneity of slopes of the calculated regression equations was performed. The best fit line for the data points considered was one where the regression equation was of the form $Y = mX + b$, where Y is the concentration of halogen and X is time, m is the slope of the regression line, and b is the Y intercept. Typical values of R^2 for these zero order descriptions were on the order of 0.95-0.99 with corresponding calculated F values exceeding the critical values of F at the 0.0001 level of significance. The one exception to these findings was the fit of the data from Compound I at pH 4.5. The interpretation of this anomaly is that there is very little effect of time on the concentration of Compound I at pH 4.5. Table 2 lists the regression equations as calculated from the high temperature stability data using the zero order and first order decay models.

As planned comparisons in the stability experiment data analysis, the slopes of the regression lines were compared for homogeneity. The calculated F values for all possible pairs of equations were found to be significant at the 0.01 probability level with the exception of the pH 7.0 and pH 9.5 curves for HTH. The calculated probability of obtaining a greater F statistic when comparing the slopes of these latter two curves was greater than 0.5. Thus, the stability curves plotted are all significantly different except the curves for HTH at pH 7.0 and 9.5.

The data in Figure 1 illustrate that Compound I is stable indefinitely in pH 4.5 demand-free water at 37°C. It is less stable at pH 7.0, 37°C (half-life of 1850 hours) than was the case for pH 7.0, 22°C (indefinite stability), but still quite a bit more stable than HTH (half-life of 625 hours) under these conditions. However, a strange reversal occurs for the two compounds at pH 9.5, 37°C; HTH becomes more stable (half-life of 775 hours) than is Compound I (half-life of 300 hours). This was not the case for pH 9.5, 22°C at which condition compound I remained more stable than HTH (12). We believe that this lack of stability of Compound I at pH 9.5, 37°C is due to decomposition of the Compound I ring rather than increased shift of the Compound I hydrolysis equilibrium to the "free chlorine" side. Such a ring degradation would be hastened by an increase in temperature. We have observed that the stability of Compound I in demand-free water at 22°C declines steadily as pH is increased, but the most dramatic decline occurs between pH 9.0 and pH 9.5.

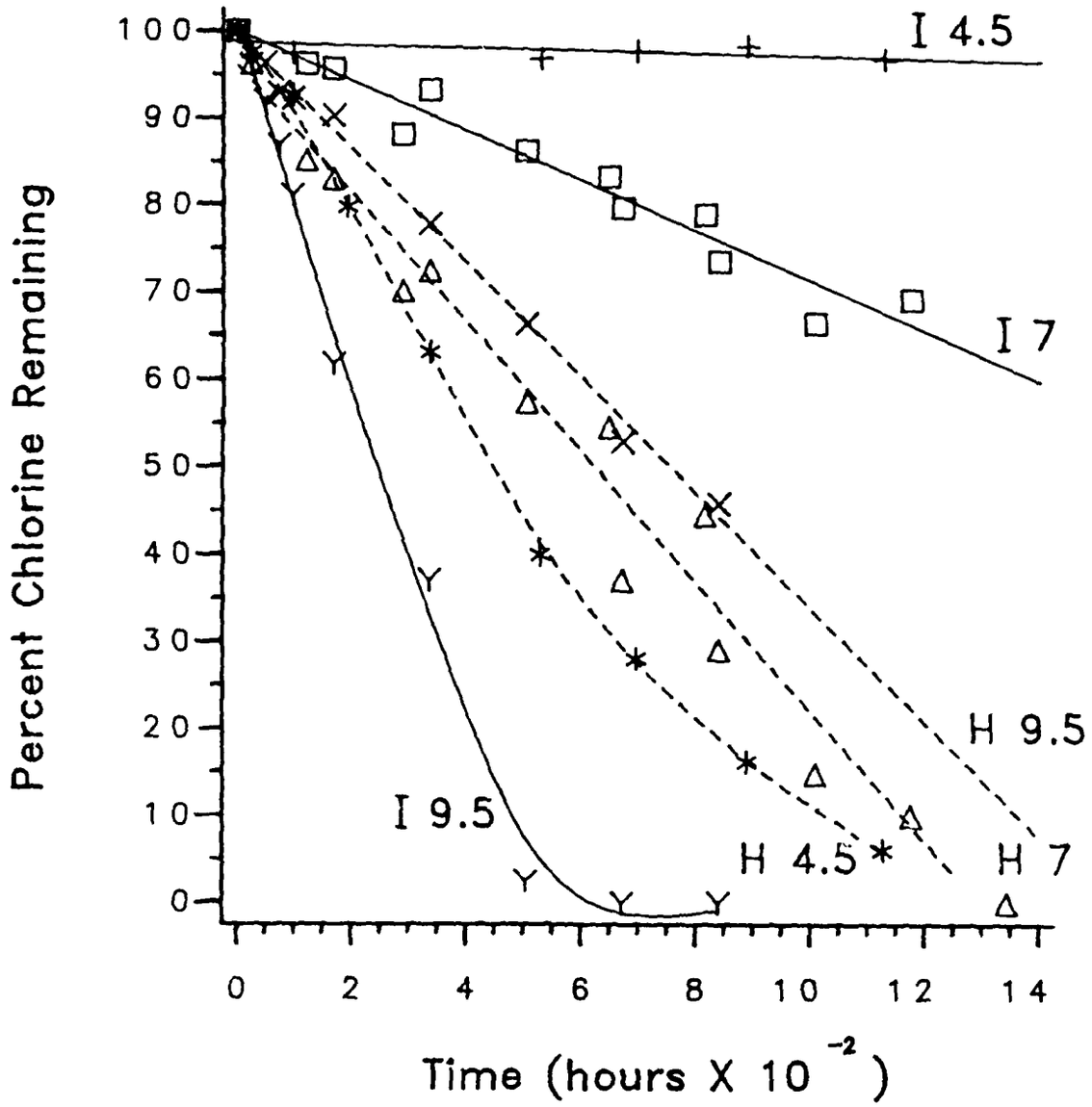


Figure 1. Stability of Compound I and HTH as a Function of pH at 37°C in Chlorine Demand-Free Water.

TABLE 2. REGRESSION EQUATIONS CALCULATED FROM 37°C STABILITY DATA

Compound	pH	Equation	Correlation Coefficient	Significance Level
HTH[a]	4.5	Y=-0.00825456X + 8.9426	0.9729	0.0001
HTH[a]	7.0	Y=-0.00682322X + 9.3278	0.9864	0.0001
HTH[a]	9.5	Y=-0.00669484X + 10.0008	0.9914	0.0001
I[a]	4.5	Y=-0.0027385X + 9.9205	0.4223	0.0580
I[a]	7.0	Y=-0.00024893 0X + 9.708	0.9596	0.0001
I[a]	9.5	Y=-0.01504817X + 9.6102	0.8732	0.0001
HTH[b]	4.5	Log Y=-0.00102214X + 1.0409	0.9961	0.0001
HTH[b]	7.0	Log Y=-0.00061963X + 1.0270	0.857	0.0001
HTH[b]	9.5	Log Y=-0.000395X + 1.0086	0.9837	0.0001
I[b]	4.5	Log Y=-0.000012X + .9965	0.4233	0.0578
I[b]	7.0	Log Y=-0.00013474X + .9895	0.9646	0.0001
I[b]	9.5	Log Y=-0.0027 0491X + 1.1319	0.8258	0.0001

a. Zero order decay model.
b. First order decay model.

The effect of ambient light on the stabilities of Compound I and HTH was evaluated at pH 7.0 in demand-free water. Solutions of the two compounds were prepared at the 10 ppm total chlorine level. One solution of each compound was exposed to room light, while a second solution of each compound was held in darkness. All flasks containing the compounds had porous cotton plugs to allow free air exchange. Temperature was not controlled in this experiment, but varied only between 23° and 27°C during the course thereof. Weekly amperometric titrations were performed.

The results of this experiment are shown in Figure 2. The decay of both Compound I and HTH was perhaps more rapid in the flasks that were left exposed to light. Subjecting the data to linear regression and analysis of covariance suggests that while the light exposed decay slopes may be different from the dark control curves, they are not significantly different. Both decay curves follow zero order kinetics where the model tested was $Y = mX + b$. The R^2 for this model was greater than 0.98 for all four decay curves, and the F value for comparison of the slopes of light versus dark treatment for each compound was not significant at the 0.05 probability level. Compound I was found to be significantly different from HTH in stability in this experiment. We conclude that ambient room light is not an important factor in the stability of either compound.

Finally, under task 6 disinfection experiments at 37°C were initiated. Compound I and HTH were compared at pH 7.0, 37°C in demand-free water as disinfectants verses S. aureus. Several total chlorine concentrations of each

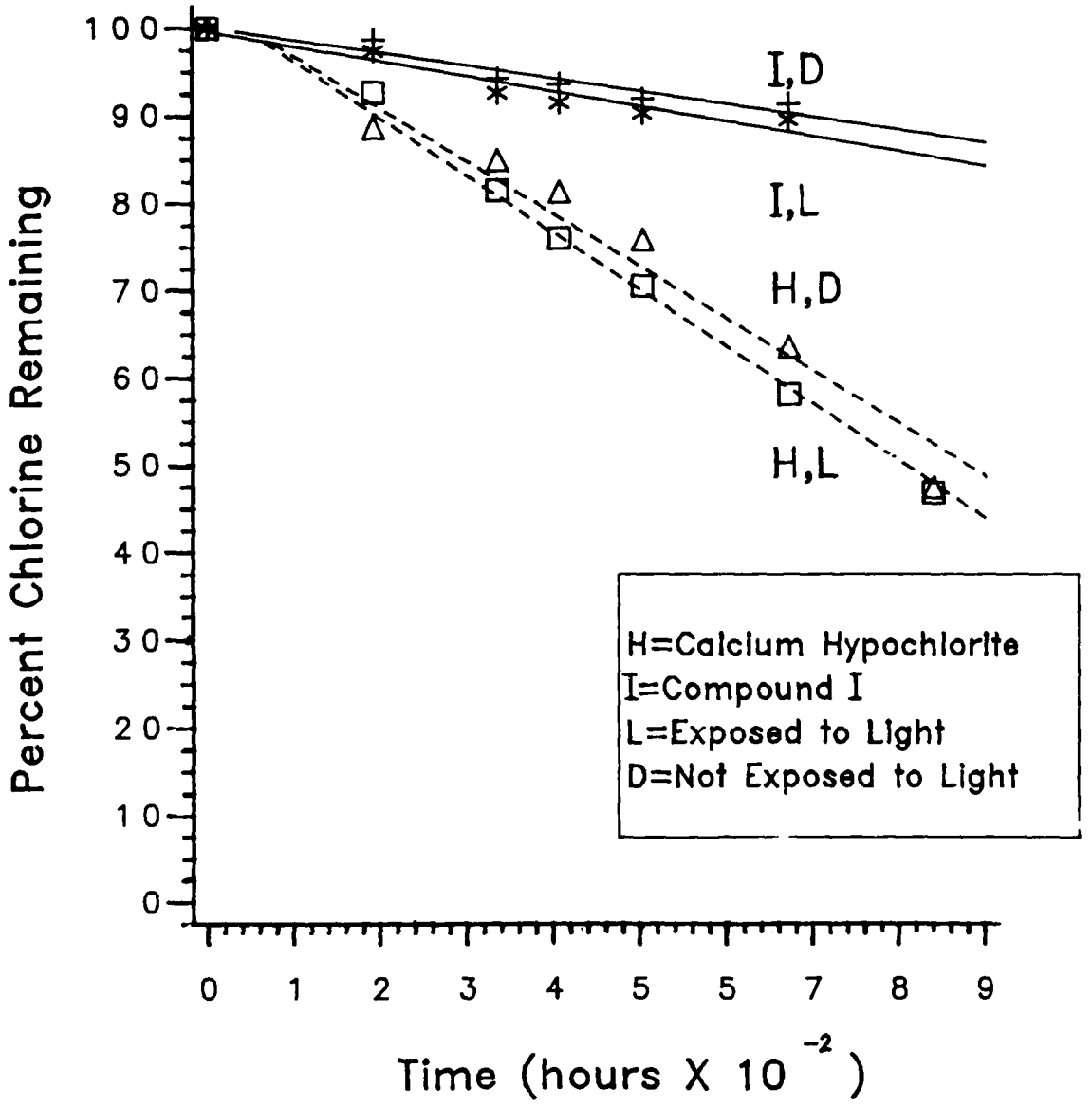


Figure 2. Stability of Compound I and HTH at pH 7.0 in the Presence of and Absence of Ambient Light.

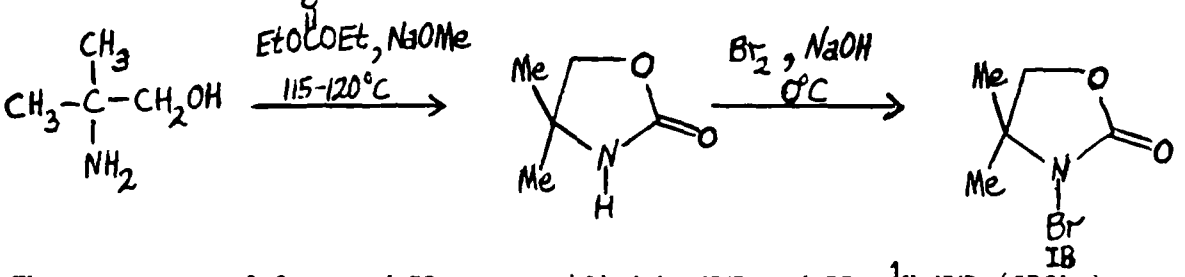
compound were used in the experiment. The disinfection c*t products for Compound I at 22°C and 37°C were 726.7 ppm-min and 94.2 ppm-min, respectively. These c*t products were obtained by multiplication of the time required for a 6 log decrease in cfu/mL calculated from a regression equation by the corresponding total chlorine concentration. The units of the products are ppm-minutes. Reliable c*t products could not be obtained for HTH at either temperature because of the rapidity of its disinfection. It is evident that compound I is a much more rapid disinfectant (8-fold) at 37°C than at 22°C for pH 7.0. This was to be expected given the rather long contact times needed for disinfection at 4°C (12). Further work needs to be done along these lines at other pH values.

SECTION III

TASK 7 (SYNTHESES OF NEW HALAMINE DISINFECTANTS)

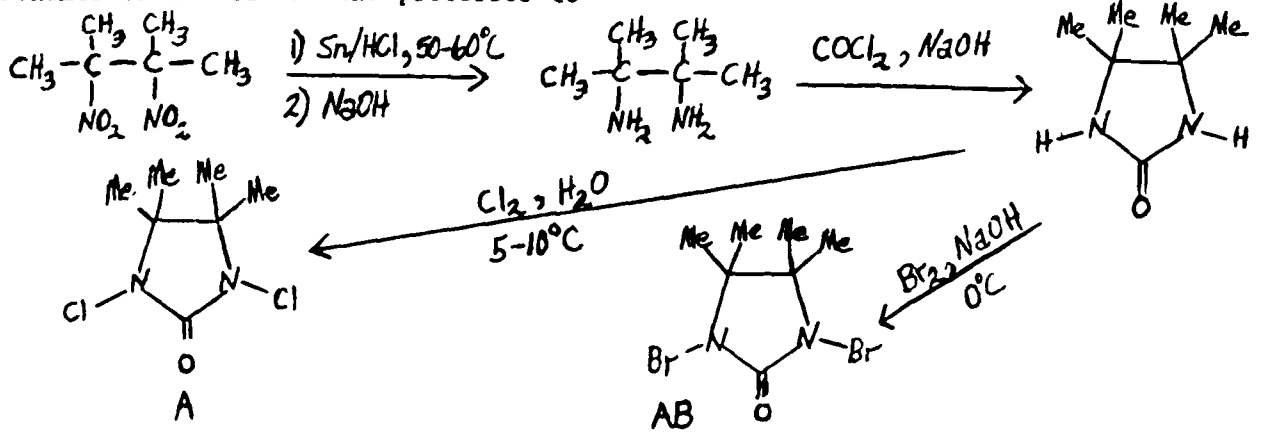
The goal of Task 7 was to synthesize Compound I and several new disinfectant compounds for testing in other tasks. The details concerning the preparation of Compound I were presented in the previous annual report (12). Those concerning the new compounds will be given in this section.

Compound IB (3-bromo-4,4-dimethyl-2-oxazolidinone) was prepared from the precursor (4,4-dimethyl-2-oxazolidinone) for Compound I by the method of Bodor and Kaminski (21). The reaction scheme is shown below.



The structure of Compound IB was verified by NMR and IR; ^{1}H NMR ($CDCl_3$): 4.27(S, 2H), δ 1.33(S, 6H); IR (KBr): 2985, 1725, 1370, 1290, 1200, 1165, 1040, 740 cm^{-1}. Compound IB is a white, crystalline solid (m.p. 118-120°C) which can be purified (to 98.6 percent) by recrystallization from carbon tetrachloride. It is also readily soluble in chloroform, methylene chloride, and methanol. Its solubility in water is 0.563 g/100 mL at 22°C, 0.310 g/100 mL at 4°C, and 0.786 g/100 mL at 32°C. The corresponding solubilities in water for Compound I were 1.282, 0.760, and 1.713 g/100 mL, respectively.

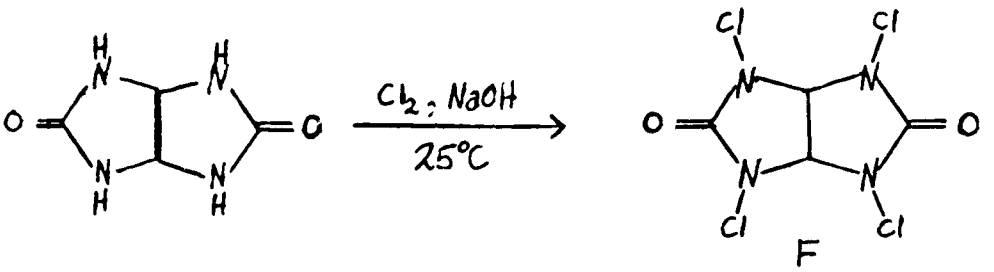
Compounds A (1,3-dichloro-4,4,5,5-tetramethyl-2-imidazolidinone) and AB (1,3-dibromo-4,4,5,5-tetramethyl-2-imidazolidinone) were prepared using the schemes shown below. The precursor to



the two disinfectants was prepared by the method of Sayre (22). Both compounds are white, crystalline solids; pertinent data are: Compound A (recrystallized from hexane to a purity of 99.8 percent; m.p. 100-102°C;

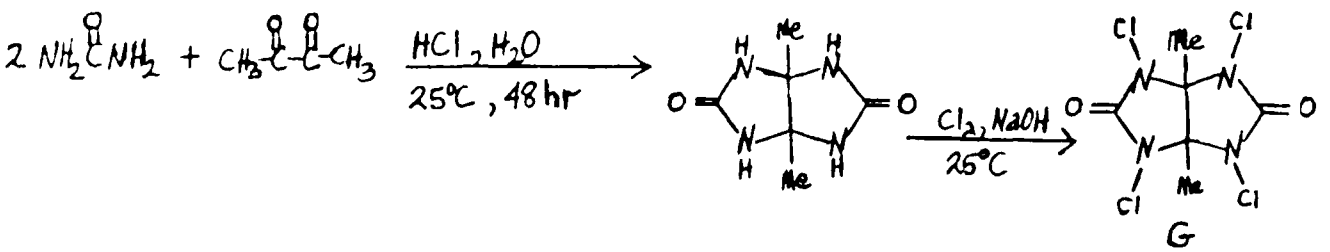
soluble in chloroform and methanol; solubility in water in g/100 mL is 0.058 at 4°C, 0.090 at 22°C, and 0.111 at 32°C; ^{1}H NMR ($CDCl_3$): δ 1.33 (S, 12H); IR(KBr): 2992, 1735, 1394, 1285, 1160 cm^{-1}; Compound AB (recrystallized from cyclohexane to a purity of 98.5 percent; m.p. 119-121°C; soluble in chloroform and methanol; solubility in water in g/100 mL is 0.130 at 4°C, 0.184 at 22°C, and 0.225 at 32°C; ^{1}H NMR ($CDCl_3$): δ 1.22 (S, 12H); IR (KBr): 2977, 1715, 1391, 1288, 1157 cm^{-1}).

Compound F (tetrachloroglycoluril) was prepared by chlorination of glycoluril which was purchased commercially.



The procedure of Slezak and coworkers (23) was employed for this chlorination. The compound was purified by recrystallization from a 2:1 benzene: ethylacetate mixture. Compound F is a white chrystalline solid having m.p 198-200°C (with decomposition). It is soluble in methanol and exhibits the following spectral data: ^{1}H NMR ($CDCl_3$): δ 5.3 (S, 2H); IR (KBr): 1772, 1372, 1170, 890, 775 cm^{-1}.

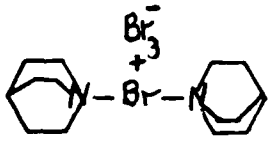
Compound G (3a,6a-dimethyl-1,3,4,6-tetrachloroglycoluril) was prepared by the scheme below. The compound was purified



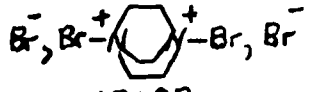
by recrystallization from benzene to 98 percent. Compound G is a white, crystalline solid with m.p. 224-226°C which is soluble in methanol; its solubility in water is 0.002 g/100 mL at 4°C, 0.005 g/100 mL at 22°C, and 0.054 g/100 mL at 32°C. Spectral data for Compound G are: ^{1}H NMR($CDCl_3$): δ 1.81 (S, 6H); IR(KBr): 3010, 1765, 1744, 1259, 1220, 1159, 730 cm^{-1}.

At the suggestion of Dr. D. Rosenblatt of USAMBRDL at Ft. Detrick the compounds QUIN (quinuclidine bromide) and DABCOB (diazabicyclooctane bromide) were prepared by the bromination of commercial quinuclidine (24) and DABCO

(25), respectively. These compounds were quite unstable and insoluble in a variety of organic solvents, and hence could not be purified by recrystallization. They were subjected to preliminary testing under Task 8.



QUINB



DABCOB

Considerable effort was also made under Task 7 to synthesize analogs of Compounds I, IB, A, and AB containing sulfur either bound in an endocyclic or exocyclic position of the rings. Our hypothesis was that sulfur might enhance the disinfection efficacy of the compounds. While the amine precursors have been prepared here, unfortunately in all attempts to date, the halogenation step caused decomposition of the cyclic systems. Work along these lines has been discontinued, at least temporarily.

Finally under Task 7, 10 g of Compound I was prepared and sent to Dr. Dinterman at USAMRIID Ft. Detrick in January 1984 for evaluation as a possible detoxicant. A 1 g sample of Compound I was sent to Dr. Noss at USAMBRDL Ft. Detrick for mechanistic studies.

SECTION IV

TASK 8 (PRELIMINARY TESTING OF NEW DISINFECTANT COMPOUNDS

The goal of Task 8 is to subject new compounds synthesized under Task 7 to preliminary screening of bactericidal efficacy and solution stability in order to provide a relatively rapid assessment of the new compounds as potential military field disinfectants. We hope to discover a new N-halamine compound which kills microorganisms at shorter contact time than Compound I, but yet which is significantly more stable in water than HTH. In fact any compound which is less stable than HTH and/or less germicidal than Compound I will not be subjected to extensive evaluation under Task 10.

The research protocols used for disinfection and stability testing under Task 8 were extensively outlined in our last annual report (12) and thus will not be discussed in detail here. For the bactricidal screening, solutions of the compounds were always subjected to $1\text{-}2\text{x}10^6$ cfu/mL of organisms. Aliquots were removed at timed intervals, quenched by 0.02 N sodium thiosulfate, and plated. Plating, colony enumeration, and data analyses procedures were performed as previously described (12). The bactericidal evaluation consisted of experiments involving Staphylococcus aureus in demand-free water at pH 7.0, 22°C at a total Cl^+ concentration (or its molar equivalent in Br^+) of 5 ppm and in "worst case water" (WCW) containing heavy organic load (12) at pH 9.5, 4°C at a total Cl^+ concentration (or its molar equivalent in Br^+) of 10 ppm; and Shigella boydii in demand-free water at pH 7.0, 22°C at a total Cl^+ concentration (or its molar equivalent in Br^+) of 2.5 ppm. Stability evaluations conducted under Task 8 included testing the compounds in demand-free water at pH 7.0, 22°C and pH 9.5, 4°C, and in WCW at pH 9.5, 4°C.

Table 3 gives test conditions and measured c*t values (see Section II) for calculated 6 log inactivation of the organisms used in the preliminary (Task 8) and extensive (Task 10) testing of the new compounds synthesized under Task 7. In most of the WCW experiments the disinfection curves were not linear, and hence the range of calculated c*t values has been given in Table 3.

The data in Table 3 indicate that all compounds tested, except Compound A, were more effective than Compound I against both S. aureus and S. boydii in demand-free water under Task 8 preliminary testing conditions. However, for worst case water test experiments this was not the case because of the great stability of Compound I under these conditions. Since Compound A was less effective than Compound I in preliminary bactericidal screening, we have decided to at least temporarily discontinue its testing. Compound IB looks particularly promising as a rapid disinfectant; this is because it forms ca. 95 percent "free bromine" in water (as measured by a DPD test kit). Figure 3 shows a comparison of its action against S. aureus in worst case water with that of Compound I. Of course compounds liberating free halogen such as IB and HTH are considerably less stable in the presence of organic load than are those which liberate little or no free halogen such as I and A (12).

TABLE 3. ACTION OF DISINFECTANTS AGAINST BACTERIA UNDER VARIOUS TEST CONDITIONS.

Organism	Conditions[a]	Compound	C*T[b] (ppm-min)
S. aureus	pH 7.0, CDF, 22 C	I	216
S. aureus	pH 7.0, CDF, 22 C	IB	3.25
S. aureus	pH 7.0, CDF, 22 C	A	1400
S. aureus	pH 7.0, CDF, 22 C	AB	9.78
S. aureus	pH 7.0, CDF, 22 C	HTH	<0.25
S. aureus	pH 7.0, CDF, 22 C	F	<1.25
S. aureus	pH 7.0, CDF, 22 C	G	<1.25
S. aureus	pH 7.0, CDF, 22 C	QUINB	<1.25
S. aureus	pH 7.0, CDF, 22 C	DABCOB	16.98
S. aureus	pH 4.5, CDF, 22 C	I	326.4
S. aureus	pH 4.5, CDF, 22 C	HTH	<1.25
S. aureus	pH 4.5, CDF, 22 C	IB	2.11
S. aureus	pH 4.5, CDF, 22 C	AB	2.44
S. aureus	pH 9.5, CDF, 22 C	I	150.8
S. aureus	pH 9.5, CDF, 22 C	HTH	4.73
S. aureus	pH 9.5, CDF, 22 C	IB	1.52
S. aureus	pH 9.5, CDF, 22 C	A	522.6
S. aureus	pH 9.5, CDF, 22 C	AB	3.28
S. aureus	pH 9.5, CDF, 4 C	I	648.5
S. aureus	pH 9.5, CDF, 4 C	HTH	24.08
S. aureus	pH 9.5, CDF, 4 C	IB	2.52
S. aureus	pH 9.5, CDF, 4 C	AB	25.13
S. aureus	WCW	I	1343-2620
S. aureus	WCW	HTH	45.65-16206
S. aureus	WCW	IB	18.5-4940
S. aureus	WCW	A	9679
S. aureus	WCW	AB	291-6270
S. aureus	WCW	F	>1200
S. aureus	WCW	G	>1200
S. boydii	pH 7.0, CDF, 22 C	I	23.39
S. boydii	pH 7.0, CDF, 22 C	HTH	<0.25
S. boydii	pH 7.0, CDF, 22 C	IB	1.29
S. boydii	pH 7.0, CDF, 22 C	A	26
S. boydii	pH 7.0, CDF, 22 C	AB	9.70
S. boydii	pH 7.0, CDF, 22 C	F	<0.625
S. boydii	pH 7.0, CDF, 22 C	G	<0.625
S. boydii	pH 9.5, CDF, 4 C	I	----
S. boydii	pH 9.5, CDF, 4 C	HTH	<2.5
S. boydii	pH 9.5, CDF, 4 C	IB	<2.5
S. boydii	pH 9.5, CDF, 4 C	AB	24.82

TABLE 3. ACTION OF DISINFECTANTS AGAINST BACTERIA UNDER VARIOUS TEST CONDITIONS (CONTINUED).

Organism	Conditions[a]	Compound	C*T[b] (ppm-min)
S. boydii	WCW	I	11.91
S. boydii	WCW	HTH	<2.5
S. boydii	WCW	IB	26.15-854
S. boydii	WCW	AB	148-314

a. CDF = demand-free water; WCW = worst case water at pH 9.5, 4°C.
b. C*T values for WCW experiments are expressed as a range due to the non-linearity of dose response in WCW.

Figures 4 and 5 show representative stability curves for all of the compounds synthesized under Task 7. These data represent the stability of these compounds in demand-free water at pH 7.0, 22°C. The stability curves for Compound I and HTH under these conditions are shown also for comparison. Compounds F, DABCOB, and QUINB were considerably less stable than was HTH. In fact DABCOB was so unstable that bromine gas was observed as it evolved from solution as the solid material was dissolved. Thus on the basis of instability, studies of compounds F, DABCOB, and QUINB have been discontinued even though all three are effective bactericides when freshly prepared.

Linear regression curve fitting indicated that Compounds A, G, HTH, and I followed first order decay kinetics marginally better than zero order. The calculated r^2 values only improved by 0.01 when the log transformation was performed to allow for first order fit. The decay curve for Compound F could not be fit to either a first order or zero order model effectively.

All of the curves presented in Figure 4 were subjected to an analysis of covariance test for homogeneity of slopes of the calculated regression lines. The model chosen to describe the stability was a first order decay of the form $\log Y = mX + b$, where Y and X are halogen concentration and time, respectively. On the basis of this model all regression lines were found to have different slopes except for compounds A and I and compounds AB and IB. The significance level chosen for these comparisons was $p = 0.01$. It is important to note that as for the high-temperature stability comparisons, these are comparisons of slopes of regression lines, not comparisons of mean concentrations.

Figure 6 presents measured stabilities of compounds IB, AB, A, G, I, and HTH in worst case water. All stability curves are not first order or even second order with respect to halogen concentration, although Compound I and AB approach a first order fit. It is our assumption that the kinetics of decomposition in synthetic demand water is a multi-order reaction that probably cannot be described by a simple decay equation. This kind of observation has been noted previously. No attempt was made to describe the decay kinetics of these compounds in worst case water.

The data in Figure 6 indicate that the half lives of compounds IB, AB, and HTH in worst case water are approximately the same. However, the

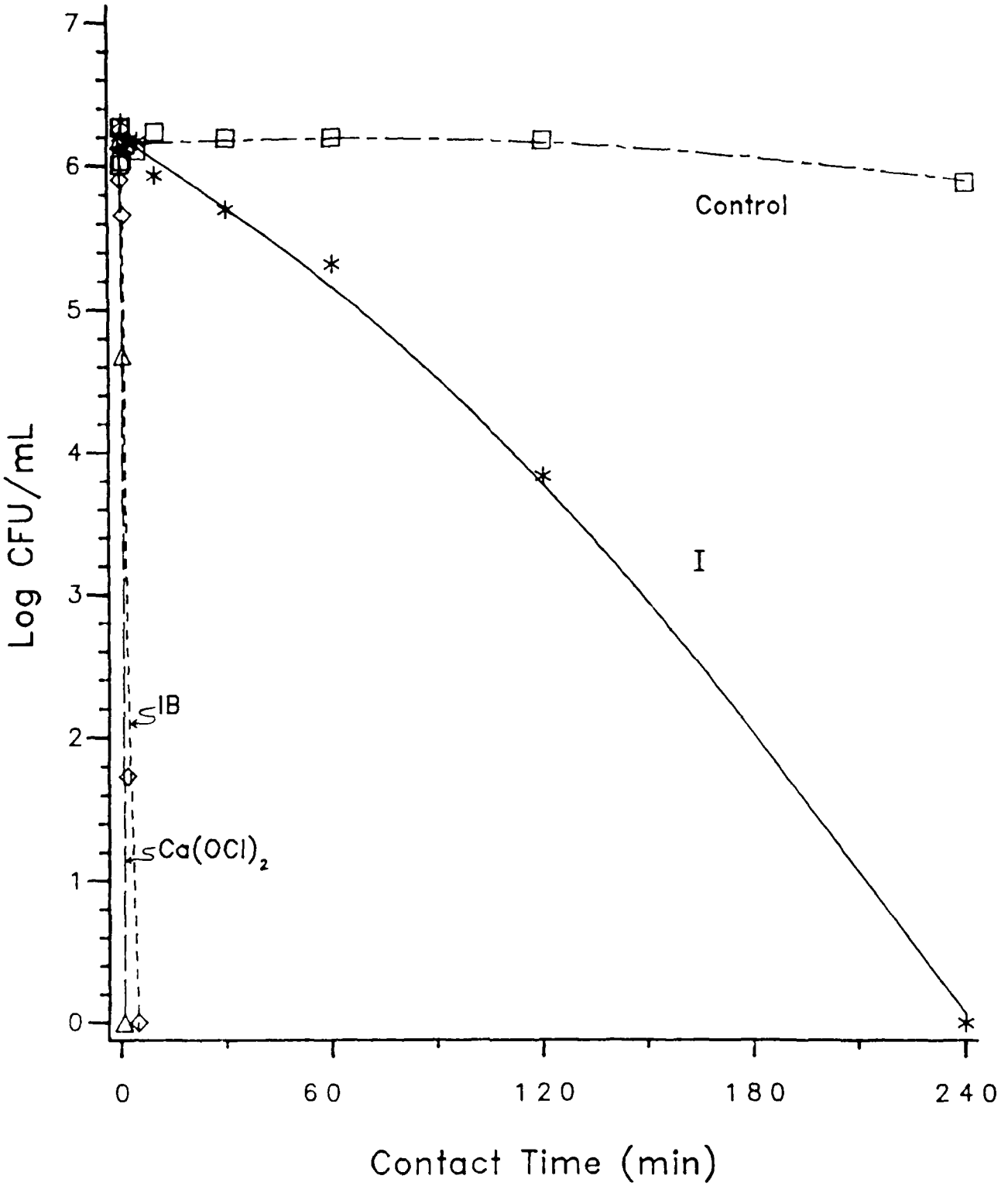
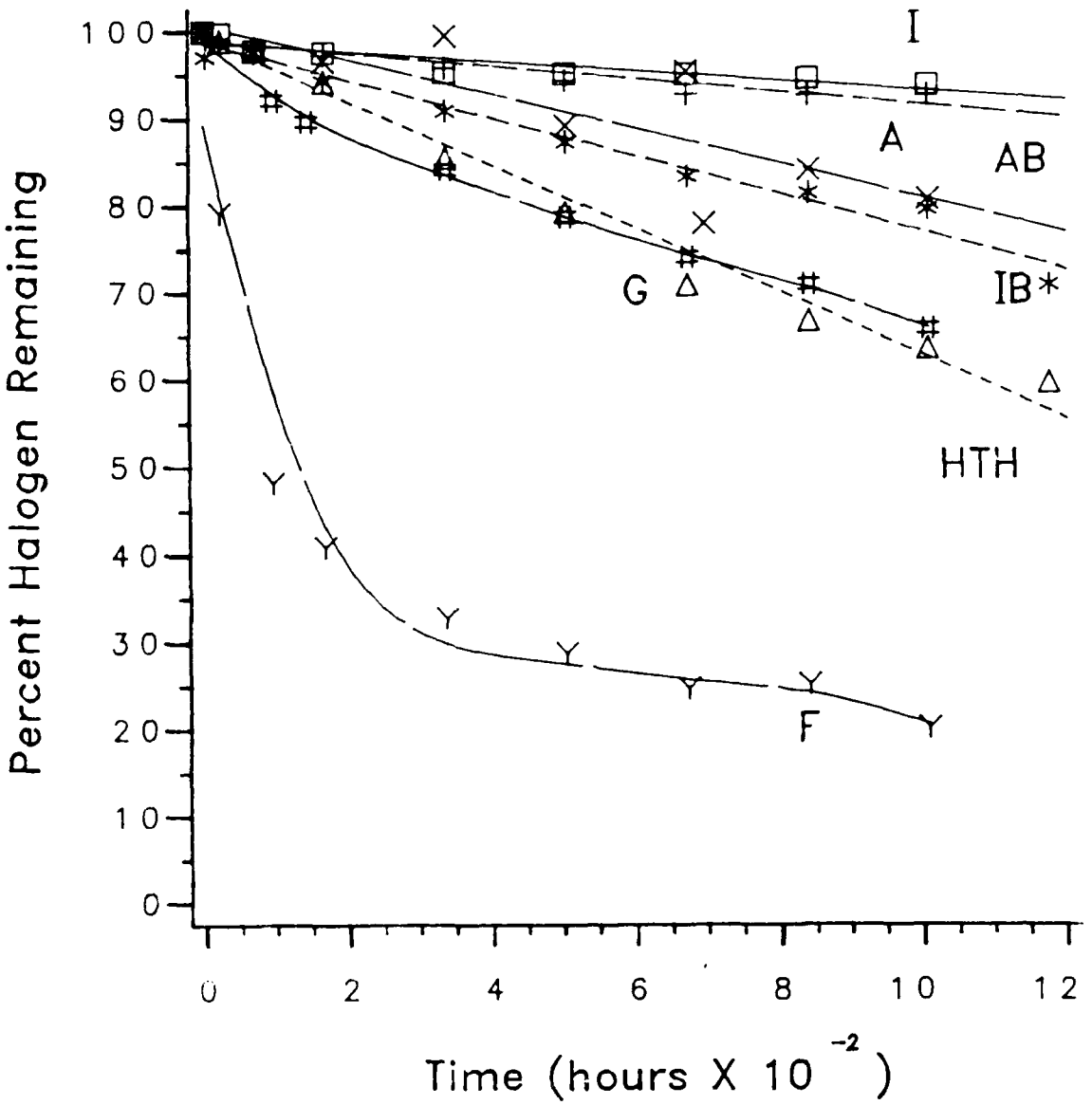
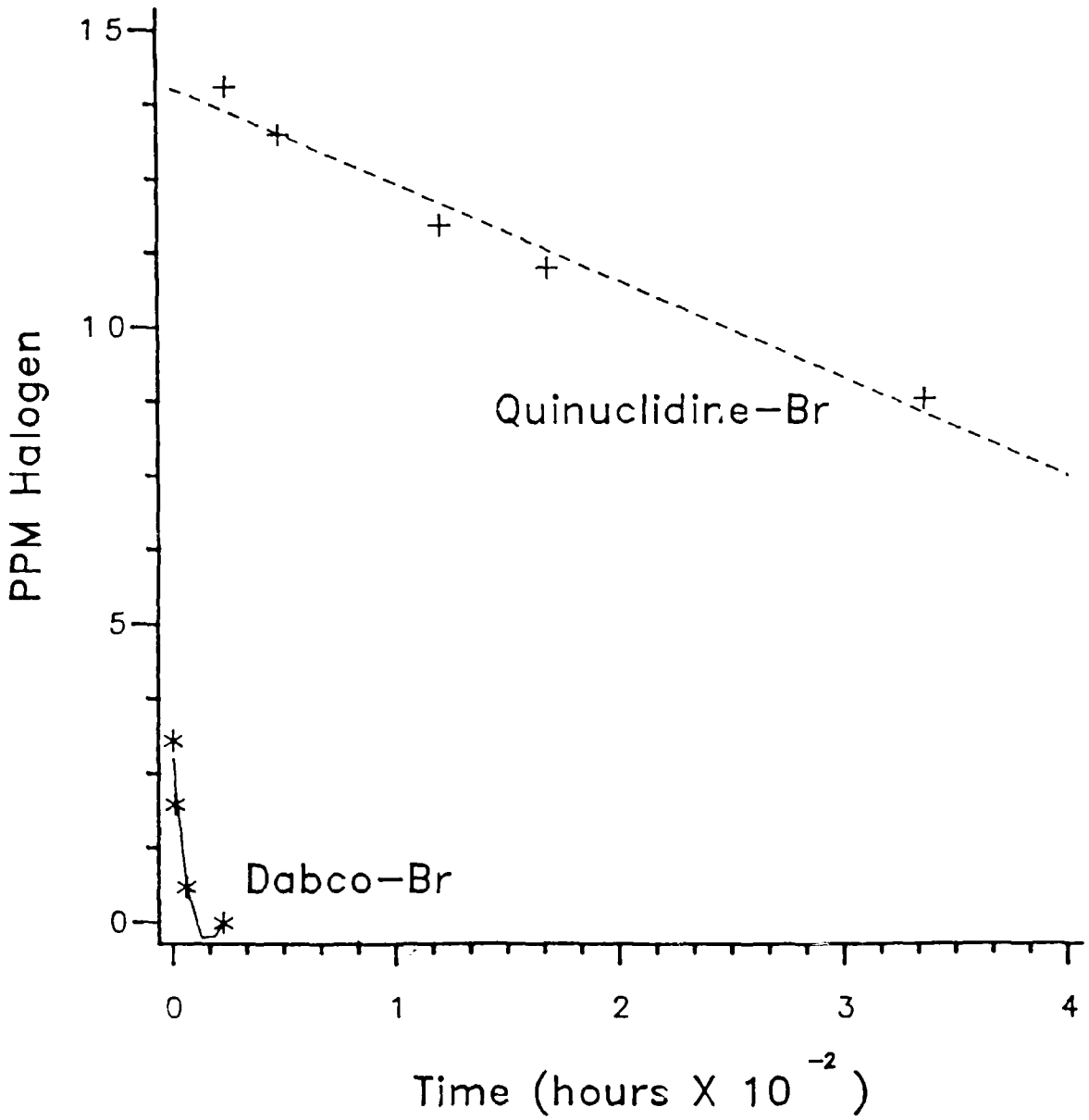


Figure 1. Comparison of the Efficacies of Compounds I, IB, and HTH as Disinfectants against Staphylococcus aureus in Worst Case Water, pH 7.5, 5°C.



[illegible] Stability of compounds I, A, AB, IB, G, F, and HTH in [illegible] water at pH 7.0, 22°C.



Stabilites of Compounds DABCOB and QUINB in Demand-Free Water at pH 7.0, 22°C.

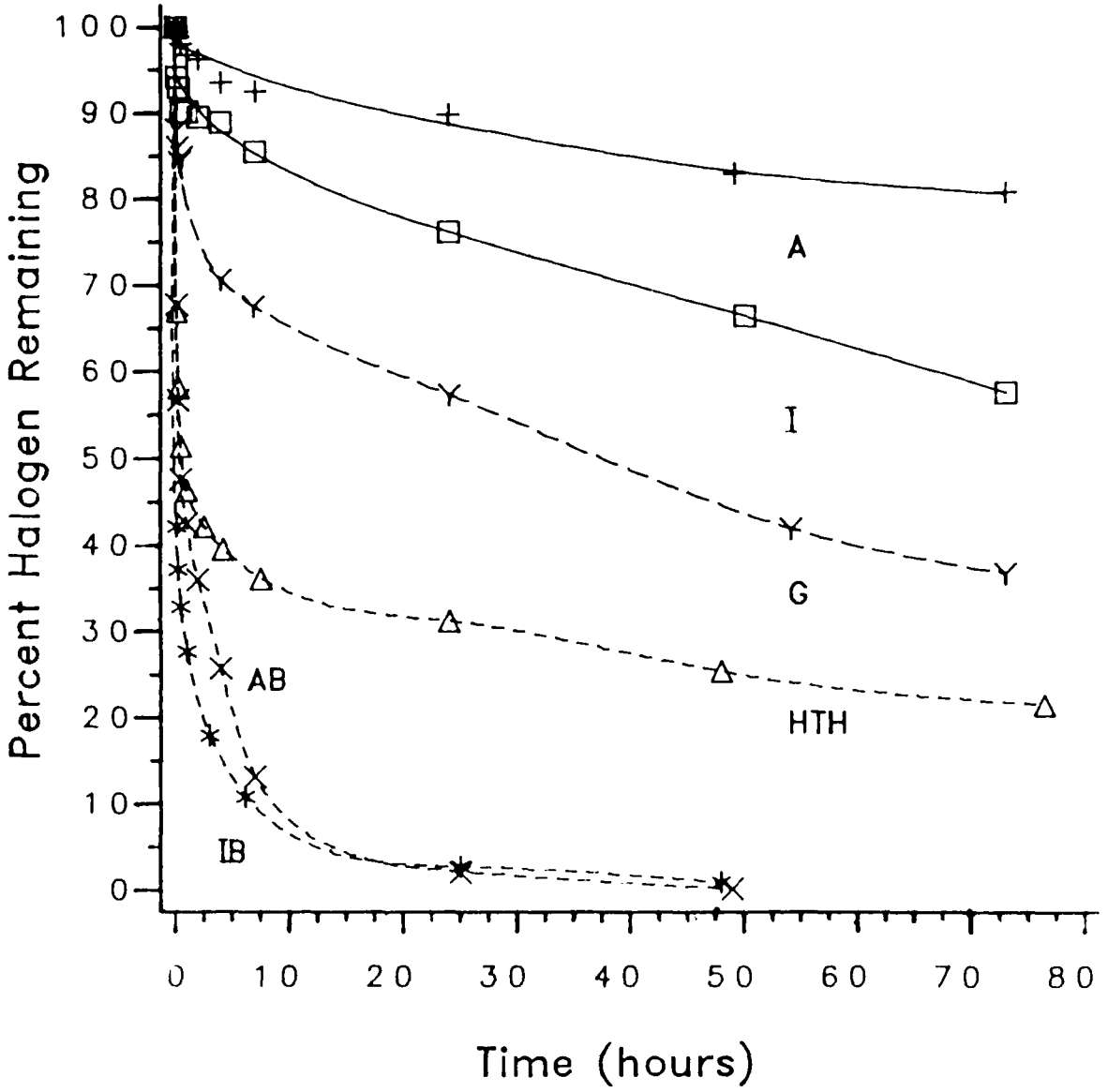


Figure 6. Stabilities of Compounds A, I, G, AB, IB, and HTH in Seven Oaks Water at pH 9.5, 5°C.

combined-halogen decomposition product from HTH is clearly more stable than those for the two N-bromamine compounds. The results obtained under Task 8 led us to subject compounds IB, AB, and G to extensive testing under Task 10.

SECTION V

TASK 9 (TESTING OF DISINFECTANT COMPOUNDS AGAINST VIRUS AND PROTOZOA SPECIES)

VIRUS STUDIES

Compound I and HTH were tested for comparative virucidal efficacy against rotavirus at the various assay conditions reported previously for studies using poliovirus type 1 (12). Compounds IB, A, and AB were tested for virucidal efficacy against both rotavirus and poliovirus at pH 7.0, 22°C. Combinations of compound I and HTH were tested for virucidal efficacy against poliovirus type 1 at pH 7.0, 22°C. The stability of the virucidal activity of combinations of compound I and HTH was determined against poliovirus type 1 by retesting at weekly intervals over a period of 4 weeks after storage of the Compound I: HTH combinations at 4°C, 25°C, or 37°C.

Virucidal studies with rotavirus: The SA-11 strain of rotavirus was acquired from the American Type Culture Collection (ATCC) (28). It was propagated in a fetal rhesus monkey kidney cell line, MA-104, which was obtained from MA Bioproducts, Inc., Walkersville, MD (29). The MA-104 cells were maintained with serum-free medium for propagating and assaying rotavirus. Stock preparations of rotavirus were harvested from infected cells by three cycles of rapid freezing and thawing, followed by centrifugation at 5,000 x g to remove particulate cell debris from the suspension of virus in cell culture fluids. The virus-containing supernatant fluids were aliquoted in 1.0 mL ampoules and stored at -70°C. Titrations were done in 96-well microtiter tissue culture plates using serial log dilutions and inoculating 0.2 mL in each of five replicate wells of MA-104 cells per dilution of virus.

Assays for virucidal effects of compound I and HTH were done in demand-free buffers at pH 7.0 and pH 9.5, and in WCW at pH 9.5. Temperature was controlled at 4°C or 22°C with a water bath in a walk-in refrigerator. The basic procedure for assays was esssentially the same as described previously for poliovirus (12). The salient aspects of the assay procedure were as follows: (1) stock rotavirus was diluted in buffer or WCW to a concentration of about 2×10^6 tissue culture infective doses ($TCID_{50}$) at a 50 percent endpoint, (2) disinfecting agent(s) was/were diluted in buffer or WCW to 2X concentration, (3) after equilibration to the assay temperature in the water bath, equal volumes of the rotavirus and 2X disinfecting agent were combined and thoroughly mixed, (4) samples were removed from the assay reaction mixture at specific time intervals and added to an equal volume of 0.02 N sodium thiosulfate as a quencher for chlorine, (5) titrations of rotavirus infectivity were done as described above; virucidal effects were evaluated based on reduction in titer compared to that in virus-buffer controls sampled at the same time intervals.

Rotavirus was more easily inactivated by Compound I than was poliovirus type 1 (Table 4). Twenty-five ppm total chlorine from Compound I caused a greater than 99.99 percent reduction in titer of rotavirus in 4 hours contact time, whereas 400 ppm were required to cause >99.99 percent reduction in titer of poliovirus in 4 hours contact time at the same conditions of assay, pH 7.0,

22°C. At pH 9.5, 22°C, 25 ppm total chlorine from Compound I effected >99.99 percent reduction in the titer of rotavirus in 2 hours contact time (Table 5). As was found with poliovirus type 1 in studies reported previously, HTH was far more efficient than Compound I at inactivation of rotavirus (Table 6). The difference between Compound I and HTH at inactivation of both poliovirus and rotavirus were so great that statistical analysis was deemed unnecessary. Virucidal activity of both Compound I and HTH was decreased by assaying at 4°C and by organic load in WCW (Tables 5 and 6). It was concluded that while HTH is superior to Compound I in terms of rapid inactivation of rotavirus at lower concentrations of total chlorine, Compound I at 25 ppm will inactivate the infectivity of rotavirus at 22°C and pH of 7.0 and 9.5 in a contact time of 4 hours. A concentration of 100 ppm total chlorine from Compound I inactivated rotavirus at all conditions of assay except pH 7.0, 4°C.

TABLE 4. VIRUCIDAL EFFICACY OF COMPOUND I AGAINST ROTAVIRUS COMPARED TO ITS EFFICACY AGAINST POLIOVIRUS TYPE 1[a]

Contact Time	Percent reduction in titer caused by ppm total chlorine against Poliovirus type 1[b]				Rotavirus[c]			
	400	100	25	10	400	100	25	10
10 min	89.70	<68.00[d]	<68.00	<68.00	<68.00	<68.00	<68.00	<68.00
1 hr	97.90	77.20	<68.00	<68.00	99.93	<68.00	<68.00	<68.00
2 hrs	99.80	77.80	70.90	<68.00	99.97	99.97	99.15	99.15
4 hrs	>99.99	88.40	68.00	<68.00	>99.99	>99.99	>99.99	99.99
24 hrs	>99.99	>99.99	97.70	95.70	ND[e]	ND	ND	ND

[a]Assay conditions were pH 7.0, 22°C.
[b]The Chat strain of attenuated poliovirus type 1 was used in the assays.
[c]The SA-11 strain of rotavirus was used in the assays.
[d]Reduction in titer by less than 0.5 $\log_{10}$ was recorded as <68.00 percent.
[e]ND = not determined.

Virucidal effects of Combinations of Compound I and HTH: Compound I and HTH were combined at 3 different ratios to yield total chlorine concentrations of either 100 ppm or 25 ppm. The ratios of Compound I to HTH were 10:1, 5:1, and 1:1. The amount of chlorine available from Compound I and HTH respectively at each of the ratios was as follows: 100 ppm total chlorine - 10:1 (90.91:9.09), 5:1 (83.33:16.67), and 1:1 (50:50); 25 ppm total chlorine - 10:1 (22.73:2.77), 5:1 (20.83:4.17), and 1:1 (12.5:12.5). The amount of chlorine available from HTH in each of the ratios for 100 ppm was great enough to effect a rapid reduction in titer of poliovirus type 1,

TABLE 6: VIRUCIDAL EFFECTS OF HTH AGAINST ROTAVIRUS (SA-11 STRAIN)

Conditions of assays			Percent reduction in titer by ppm total chlorine[a]			
Time	pH	Temp.	25	10	5	2.5
10 min	7.0	4 C	>99.99	46.80	21.40	0
	7.0	22 C	>99.99	>99.99	90.00	0
	9.5	22 C	>99.99	>99.99	99.00	21.40
	9.5	4 C	>99.99	99.56	32.00	21.40
(WCW)	9.5	4 C	99.22	91.93	15.00	0
30 min	7.0	4 C	>99.99	90.00	46.80	0
	7.0	22 C	>99.99	>99.99	91.07	21.40
	9.5	22 C	>99.99	>99.99	99.00	21.40
	9.5	4 C	>99.99	99.92	59.05	32.00
(WCW)	9.5	4 C	99.92	96.77	32.00	23.00
1 hr	7.0	4 C	>99.99	99.00	46.80	21.40
	7.0	22 C	>99.99	>99.99	90.00	21.40
	9.5	22 C	>99.99	>99.99	99.21	46.80
	9.5	4 C	>99.99	>99.99	92.35	39.40
(WCW)	9.5	4 C	>99.99	99.06	58.95	32.00
2 hr	7.0	4 C	>99.99	99.90	90.00	21.40
	7.0	22 C	>99.99	>99.99	91.22	34.10
	9.5	22 C	>99.99	>99.99	99.92	21.40
	9.5	4 C	>99.99	>99.99	94.90	59.05
(WCW)	9.5	4 C	>99.99	99.93	90.75	50.20
4 hr	7.0	4 C	>99.99	99.90	90.00	46.80
	7.0	22 C	>99.99	>99.99	99.45	46.80
	9.5	22 C	>99.99	>99.99	99.99	90.00
	9.5	4 C	>99.99	>99.99	99.92	90.54
(WCW)	9.5	4 C	>99.99	>99.99	92.78	58.95

[a] Numbers for percent reduction in titer are averages of three or four separate assays done on different days or the results of two assays for which the results were identical.

whereas the amount of chlorine available from Compound I was enough to effect >99.99 percent reduction in titer between 4 and 24 hours of contact time (Table 7). Only the 1:1 ratio in 25 ppm total chlorine assays had adequate HTH to effect a rapid reduction in titer of poliovirus, and the amount of Compound I was not adequate to effect a significant reduction in titer of poliovirus (Table 7). The ratios and total amounts of chlorine used enabled determinations on the stability of each component at three different storage temperatures by retesting at weekly intervals.

Stability of virucidal activity of combinations of Compound I and HTH at different storage temperatures: Each of the three ratios of mixtures of Compound I and HTH were assayed immediately after the mixtures were prepared. The remaining portion of each ratio at the two different concentrations of total chlorine were placed in tightly capped, chlorine demand-free glass bottles for storage. One portion of each ratio was placed in a refrigerator at 4°C, a second portion was stored at room temperature in the laboratory at approximately 25°C, and the third portion was stored in a 37°C incubator. At weekly intervals over a period of 4 weeks, aliquots were removed from each storage container, and the virucidal activity was assayed against poliovirus type 1 as reported previously (12). There was no change in the virucidal activity of the samples stored at 4°C over a period of 4 weeks (Table 7). The virucidal activity of the samples stored at 25°C and at 37°C decreased markedly beginning the second week after preparation of the mixtures. The pattern of virus inactivation from the second through the fourth week of storage was typical of virucidal effects of Compound I (Table 7). The results suggested that the virucidal activity from the free chlorine of HTH had undergone degradation at storage temperatures of 25°C and 37°C. The 1:1 ratio mixture with 100 ppm total chlorine was the only mixture that inactivated the virus significantly in less than 10 minutes when assayed after two weeks of storage at either 25°C or 37°C. These results suggest that the chlorine from HTH had decayed to less than 9 ppm since that was the lowest concentration of chlorine from HTH, 10:1 ratio, in the assays that effectively inactivated the poliovirus in less than 10 minutes (Table 7). The results of these assays agree with results reported previously from our laboratory on virucidal effects of HTH against poliovirus type 1 in which 10 ppm chlorine from HTH rapidly inactivated poliovirus, but 5 ppm did not significantly kill the same virus when assayed at pH 7.0, 22°C (12).

Virucidal Activity of Synthetic N-halamine Compounds IB, AB, and A Compared to Compound I: Standard assays as described above and as reported previously were done against poliovirus type 1 (Chat strain) and rotavirus SA-11 to compare the virucidal activity of newly synthesized N-halamine compounds with Compound I. All assays were done at pH 7.0, 22°C. Two or three separate assays were done for each compound except Compound A which was found to have little or no virucidal properties under the conditions tested. Compound IB was more effective than Compound I against both poliovirus and rotavirus (Table 8). Compound AB was similar to Compound I in virucidal activity against poliovirus, but it was slightly more effective against rotavirus than Compound I (Table 8). None of these N-halamine compounds approached the virucidal efficacy of HTH.

TABLE 7: THE EFFECT OF STORAGE TEMPERATURE ON VIRUCIDAL PROPERTIES OF RATIO MIXTURES OF COMPOUND I & HTH AGAINST POLIOVIRUS TYPE 1

Storage Conditions		Time for 99.99% reduction in titer of poliovirus[a]					
		100 ppm total chlorine[b]			25 ppm total chlorine[b]		
Temp.	Duration	10:1	5:1	1:1	10:1	5:1	1:1
Mixtures - time 0		<10[c]	<10	<10	*	*	<10
4 C	1 week	<10	<10	<10	*	*	<10
	2 weeks	<10	<10	<10	*	*	<10
	3 weeks	<10	<10	<10	*	*	<10
	4 weeks	<10	<10	<10	*	*	<10
25 C	1 week	<10	<10	<10	**	*	<10
	2 weeks	*	*	<10	**	****	***
	3 weeks	*	*	*	****	****	****
	4 weeks	*	*	*	****	****	****
[illegible] C	1 week	<10	<10	<10	***	*	<10
	2 weeks	*	*	<10	**	****	***
	3 weeks	*	*	*	****	****	****
	4 weeks	*	*	*	****	****	****

[a] All assays were run at pH 7.0, 22 C; samples were collected after contact times of 10 min, 30 min, 1 hour, 2 hrs, 4 hrs, and 24 hrs.

[b] Compound I and HTH were combined at 3 different ratios to yield total chlorine of either 100 or 25 ppm. At 100 ppm, the chlorine available from cmpd I & HTH respectively at each ratio was: 10:1 (90.91:9.09), 5:1 (83.33:16.67), & 1:1 (50:50); at 25 ppm: 10:1 (22.73:2.77), 5:1 (20.8:4.17), & 1:1 (12.5:12.5).

[c] <10 = less than 10 minutes contact time for >99.99% reduction in titer.

* = >99.99% reduction in titer in 24 hours, <90% reduction at 4 hours.

** = >99.99% reduction in titer in 24 hrs, <90% reduction at 4 hours.

*** = >99.99% reduction in titer in 24 hrs, <90% reduction at 4 hours.

**** = >99.99% reduction in titer in 24 hrs, <90% reduction at 4 hours.

TABLE 8: COMPARISON OF SYNTHETIC N-HALAMINE COMPOUNDS FOR VIRUCIDAL ACTIVITY AGAINST POLIOVIRUS TYPE 1 AND ROTAVIRUS SA-11[a]

ppm tested of halogen	Time in hours for >99.99% reduction in titer of virus[b] Poliovirus type 1 I[a]	IB	AB	A	Rotavirus SA-11 I[a]	IB	AB	A
400	2-4[b]	ND[c]	ND	ND	2-4	ND	ND	ND
200	ND	½-1	ND	>24[b]	ND	1-2	ND	>4[b]
100	4-24	2-4	4-24	>24	2-4	2-4	1-2	>4
50	ND	ND	4-24	>24	ND	ND	2-4	>4
25	>24	>24	>24	>24	2-4	2-4	>4	>4
10	>24	>24	>24	>24	>4	>4	>4	>4

[a] Synthetic N-halamine compounds I, IB, AB, and A were tested. All assays were run at pH 7.0, 22 C; samples were collected at contact times of 10 min, 30 min, 1 hour, 2 hrs, 4 hrs, and 24 hrs; 4 hrs was the last sampling time for rotavirus assays.

[b] The first number in columns is contact time when <99.99%; second number is next sampling time when >99.99% reduction in titer had had occurred; therefore, numbers in columns indicate time interval during which >99.99% reduction in titer occurred; >4 or >24 means that reduction in titer was <99.99% at last sampling time.

[c] No determination.

PROTOZOA STUDIES

In the previous report (12) Compound I and HTH had been tested against Entamoeba invadens and Giardia lamblia in demand-free buffers at pH 7.0 and pH 9.5 and at temperatures 4°C and 22°C; Compound I was more efficient than HTH at killing both protozoa tested. Results were not complete at that time for assays run in worst case water (WCW) at pH 9.5. The results of assays run at pH 9.5, 4°C in WCW with Compound I and HTH against both protozoa are presented in this report along with data from assays run in buffer at pH 9.5, 4°C. Results are presented from studies done on the stability of protozoacidal activity of mixtures of Compound I and HTH after storage at 4°C and 37°C for 4 weeks.

The procedures for assays of protozoacidal activity of Compound I and HTH were described previously (12). Because of the complexity of precise quantitation, results were recorded based on a subjective assessment of viability, or lack of viability, from observations of growth in medium after treatment with disinfecting agents, compared to the growth of cultures processed from buffer controls in the assay procedures. The method of recording the results was reported previously, but is presented below for reference to the results in this report:

(+) - growth of organisms essentially the same as those of buffer controls.

(+-) - greater than 90% reduction in growth of viable organisms compared to buffer controls, subjective assessment.

(-) - no evidence of growth of viable organisms treated with Compound I or HTH compared to growth of organisms from buffer controls, subjective assessment.

As presented in Table 9, the organic load and increased mineral content of WCW had a greater effect on the efficacy of HTH than the efficacy of Compound I at killing either E. invadens or G. lamblia. Compound I was more efficient than HTH at rendering both protozoa nonviable under all conditions of assays. Complete killing of G. lamblia in less than 2 minutes by 1 ppm Compound I at pH 9.5, 4°C in demand-free buffer, and complete killing in WCW at pH 9.5, 4°C in 2 minutes by 10 ppm Compound I, in 5 minutes by 5 ppm, and in 10 minutes by 2 ppm, indicates a high degree of efficiency of Compound I at killing Giardia.

The efficiency of killing Giardia lamblia in our studies is better than that reported by others (30). The complete killing of Giardia by HTH in less than 1 min by 5 ppm chlorine and by 2 ppm within 5 minutes in demand-free buffer at pH 9.5, 4°C is similar to the efficiency reported previously at pH 7.0, 5°C and pH 9.5 at 22°C (12). However, it was reported by others that 30 minutes contact was required for 8 ppm chlorine from sodium hypochlorite to kill giardial cysts at 5°C, pH 6 and 7, and that 30 minutes contact was required for complete killing of giardial cysts at pH 8 (30). One difference that may account for the more efficient results in our studies is

TABLE 9: COMPARISON OF COMPOUND I AND HTH FOR PROTOZOACIDAL ACTIVITY AGAINST *ENTAMOEBA INVADENS* AND *GIARDIA LAMBLIA*[a]

Contact Time	Assay Medium	Compound I - ppm Cl^+ 20	10	5	2	1	HTH - ppm Cl^+ 20	10	5	2	1
Entamoeba invadens											
2 min	buffer	-[b]	-	-	+[b]	+	-	±[b]	±	+	+
	WCW	-	-	+	+	+	+	+	+	+	+
5 min	buffer	-	-	-	±	+	-	-	±	+	+
	WCW	-	-	±	+	+	±	+	+	+	+
10 min	buffer	-	-	-	±	+	-	-	±	+	+
	WCW	-	-	-	±	+	-	±	+	+	+
Giardia lamblia											
2 min	buffer	-	-	-	-	-	-	-	-	±	+
	WCW	-	-	±	+	+	-	±	+	+	+
5 min	buffer	-	-	-	-	-	-	-	-	-	±
	WCW	-	-	-	+	+	-	±	+	+	+
10 min	buffer	-	-	-	-	-	-	-	-	-	±
	WCW	-	-	-	-	+	-	-	+	+	+

[a] Assays were run at pH 9.5, 4 C in buffer or WCW, results represent average of 3 or more assays run on different dates.

[b] (+) = growth of organisms essentially the same as those of controls.
(±) = >90% reduction in growth of viable organisms compared to controls.
(-) = no evidence of growth or viable organisms, complete inactivation.

the biophysical form of the Giardia being treated. It is possible that we have been treating arrested trophozoites of Giardia lamblia rather than true cysts as reported by others (30). The growth characteristics of the supposed cysts after they were put back into medium suggest that what were thought to be cysts morphologically in our studies were, in fact, arrested trophozoites morphologically resembling cysts. Giardial cysts will not excyst and grow in medium without first being exposed to proteolytic enzymes and pH of 2 or less.[31,32] Therefore, comparison of our results with findings of others may not be completely valid. Because of this possible difference in the treatment of arrested trophozoites, rather than true giardial cysts, studies have been initiated to purify cysts of Giardia lamblia from feces of infected animals and to use purified giardial cysts in assays instead of cultures of Giardia lamblia trophozoites that are maintained in culture in the laboratory.

Protozoacidal activity of Combinations of Compound I and HTH: Compound 1 and HTH were combined at three different ratios to yield total available chlorine of 5 ppm. The ratios of Compound 1 to HTH were 9:1, 5:1, and 1:1 with the amount of chlorine available from Compound I and HTH, respectively, at each ratio as follows: 9:1 (4.5:0.5), 5:1 (4.17:0.83), and 1:1 (2.5:2.5). Dilutions were made from each of the ratio mixtures to yield total chlorine concentrations of 5, 2, 1, 0.5, and 0.1 ppm for assays immediately after preparation of the ratio mixtures. The remainder of each of the ratio mixtures at 5 ppm total chlorine was divided into 2 samples which were placed in tightly capped chlorine-demand free glass bottles for storage at 4°C or 37°C. After 4 weeks of storage, each of the ratio mixtures was diluted to yield total chlorine concentrations of 5, 2, 1, 0.5, and 0.1 ppm for assays of protozoacidal activity against both Entamoeba invadens and Giardia lamblia.

There was no decrease in protozoacidal activity after 4 weeks storage at 4°C and only a slight change in activity after 4 weeks storage at 37°C (Table 10). Since Compound I is more effective than HTH at killing both Entamoeba and Giardia, the stability of the protozoacidal activity was anticipated. The time required for killing of either Giardia or Entamoeba at the concentrations of chlorine tested suggested that the effects were primarily due to Compound I kinetics with little evidence of synergism between Compound I and HTH. The high efficiency of killing of protozoa by Compound I and its stability at temperatures ranging from 4°C to 37°C indicate that Compound I holds great promise for disinfecting water contaminated with either Giardia or Entamoeba.

TABLE 10: STABILITY OF PROTOZOACIDAL ACTIVITY OF RATIO MIXTURES OF COMPOUND I AND HTH[a]

Ratio of Cmpd I:HTH	Total Cl^+ in assay	Time in minutes for complete killing[b] Giardia lamblia			Entamoeba invadens		
		Time 0	4 C	37 C	Time 0	4 C	37 C
9:1	5 ppm	<2	<2	<2	<2	<2	<2
	2 ppm	<2	<2	<2	<2	<2	<2
	1 ppm	<2	<2	<2	5-10	5-10	>10
	.5 ppm	>10	>10	>10	>10	>10	>10
	.2 ppm	>10	>10	>10	>10	>10	>10
5:1	5 ppm	<2	<2	<2	<2	<2	<2
	2 ppm	<2	<2	<2	<2	<2	2-5
	1 ppm	<2	<2	2-5	>10	>10	>10
	.5 ppm	>10	>10	>10	>10	>10	>10
	.2 ppm	>10	>10	>10	>10	>10	>10
1:1	5 ppm	<2	<2	<2	<2	<2	<2
	2 ppm	<2	<2	<2	5-10	5-10	>10
	1 ppm	5-10	5-10	>10	>10	>10	>10
	.5 ppm	>10	>10	>10	>10	>10	>10
	.2 ppm	>10	>10	>10	>10	>10	>10

[a] Ratio mixtures of compound I and HTH were assayed immediately after preparation and again after storage for 4 weeks at either 4 or 37 C.
Time 0 column = results of assays immediately after preparation.
4 C column = results of assays after 4 weeks storage at 4 C.
37 C column = results of assays after 4 weeks storage at 37 C.

[b] Samples were collected at contact times of 2, 5, and 10 minutes.
<2 = complete killing in sample collected at 2 minutes.
2-5 = incomplete killing in sample collected at 2 minutes, complete killing in sample collected at 5 minutes.
5-10 = incomplete killing in sample collected at 5 minutes, complete killing in sample collected at 5 minutes.
>10 = incomplete killing in sample collected at 10 minutes.

SECTION VI

TASK 10 (EXTENSIVE TESTING OF PROMISING NEW DISINFECTANT COMPOUNDS)

The goal of Task 10 is to subject those new compounds found to be promising candidates for military field disinfectants under preliminary testing in Task 8 to more extensive testing as bactericides and in terms of stability as a function of pH, temperature, and water quality.

The research protocol for the extensive testing procedures under Task 10 has been outlined in the previous annual report (12). Essentially Task 10 is just an extension of Task 8. The action of the promising disinfectants against Staphylococcus aureus, which has been the most resistant bacterium tested here to Compound I disinfection, has been studied for demand-free water at pH 7.0, 22°C at 5, 2.5, and 1 ppm total Cl^+ (or its molar equivalent in Br^+); at pH 9.5, 22°C at 5, 2.5, and 1 ppm total Cl^+ (or its molar equivalent in Br^+); at pH 9.5, 4°C at 5 and 2.5 ppm. Disinfection of Shigella boydii was also studied for demand-free water at pH 7.0, 22°C at 2.5 and 1 ppm; at pH 9.5, 4°C at 5 and 2.5 ppm; and in worst case water at pH 9.5, 4°C at 5 and 2.5 ppm. Some testing will be done at a later time for the organisms in demand-free water at pH 4.5, 22°C.

Dose-response plots for time required for a 6 log kill of S. aureus by Compounds IB and AB at pH 7.0, 22°C and pH 9.5, 22°C are given in Figure 7. The data in Figure 7 show that: (1) both IB and AB are much more rapid disinfectants against S. aureus at a given concentration than is Compound I (20 minutes contact required at 10 ppm Cl^+ (12)); (2) Compound IB is superior to Compound AB for disinfection of S. aureus; and (3) both compounds disinfect more rapidly at pH 9.5 than at pH 7.0. The latter observation was made also for Compound I (12).

The other results of bactericidal testing under Task 10 are shown in Table 3 (Section IV, page 20). Again, the data in Table 3 are presented as c*t products (concentration in ppm, time in minutes). The lower the c*t product, the better was the disinfectant under the test conditions employed. It is clear that compounds IB, AB, and G perform very well as disinfectants relative to Compound I, and in some cases even better than HTH, particularly at low temperature and in alkaline medium. Compounds IB and AB work well in worst case water, although they are considerably less stable than Compound I in WCW.

In an attempt to further refine the mathematical description of the disinfection experiments, several regression models were tested using the General Linear Models procedure of SAS. The model used for construction of c*t tables and for the previous year's calculations of projected disinfection times was one for which the "log + 1" transformation (20) was applied to the survival data. The regression model arising from this transformation is one for which $\log_{10}(N/N_0 + 1)$ is the dependent variable and measured contact time (time) is the independent variable. This model was able to describe greater than 80 percent of the variation in the pooled data for each treatment condition. In an attempt to define the kinetic model more closely, several other dependent variables were fit to a linear model:

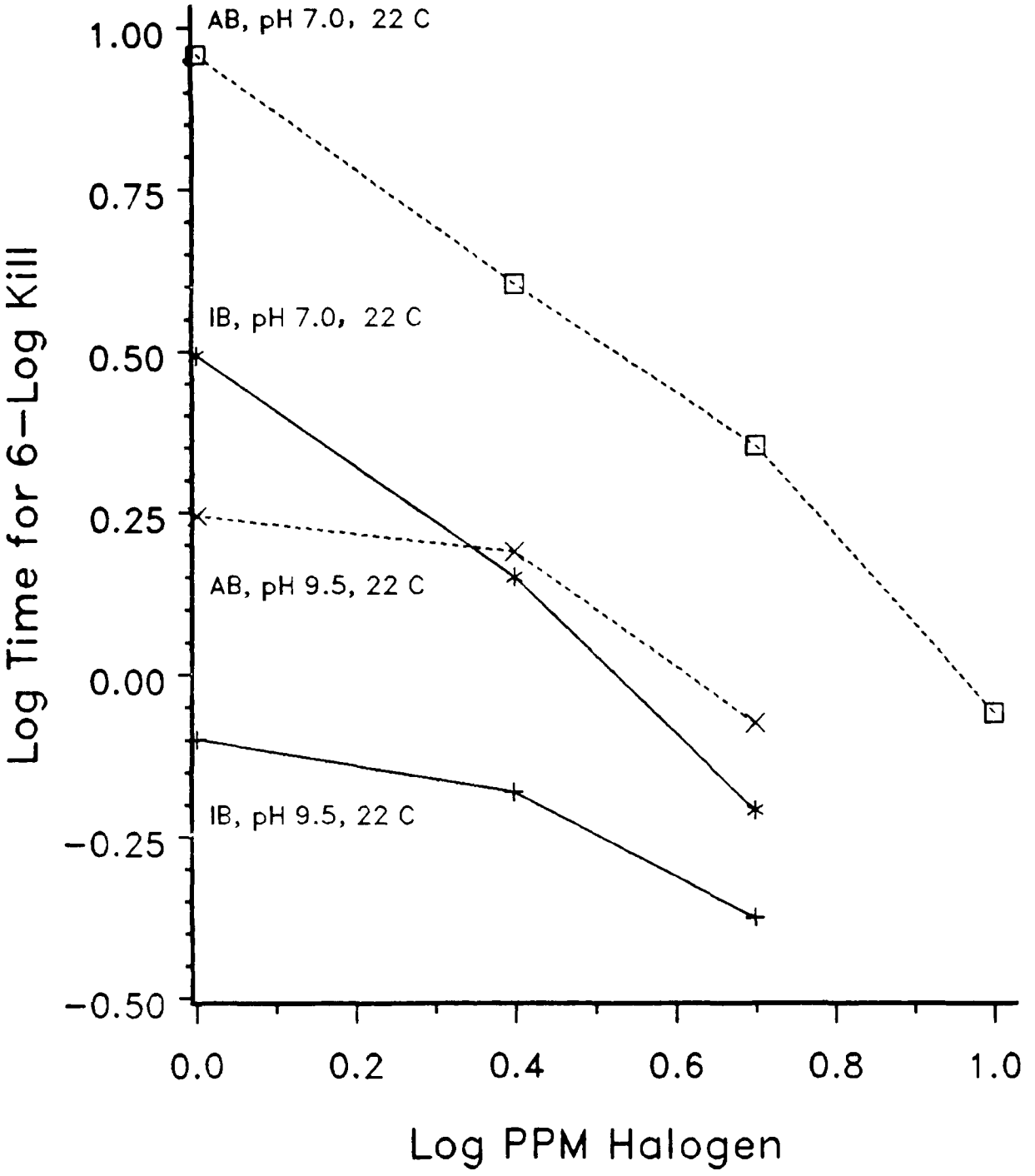


Figure 7. Dose Response for Compounds IB and AB against S. aureus. Halogen Concentrations were the Molar Equivalents of 1, 2.5, 5, 10 PPM Cl^-; Time was Expressed as Minutes.

cfu/mL = Time

N/N_0 = Time where N is cfu/mL at time t, and N_0 is cfu/mL at time 0

$\ln(N/N_0)$ = Time

ln of the arc sine transformation of N/N_0 = Time (see reference 20)

The last three models listed use the ratio of survivors and transformation of that ratio, the same as in the often used Chick-Watson Model (26,27,33,34) without the constants k and C. The natural log of the arc sine transformation of the survivor ratio seems to best describe the inactivation kinetics encountered in our experiments, although the more simple log + 1 transformation approaches the arc sine transformation based on maximizing R^2 and the F values. Unfortunately, previously collected data cannot be analyzed using the log arc sine transformation of (N/N_0) because the first time point taken in the first year's experiments was at 15 seconds contact time rather than a true time zero. Therefore, our comparisons of c*t values will have to be made using the already calculated disinfection times from the log(cfu + 1) model.

An analysis of covariance was conducted on the calculated disinfection rates using both the "log + 1" transformation model and the arc sine transformation to test whether the two bromamines had significantly different rates of disinfection. In all cases except for experiments for which the rate of disinfection was too rapid to be measured and two worst case water experiments for which the disinfection curve could not be fit to a linear model, the rates of disinfection by Compound IB were different from that of Compound AB. The significance level used for the comparisons was 0.001.

The results of stability testing of Compound G in demand-free water at pH 4.5, 7.0, and 9.5 at 22°C are shown in Figure 8. All curves are first order with respect to total Cl^+ concentration and could be fit to a model of the form:

$$\log Y = mX + b.$$

Tests for slope homogeneity using this model indicated that the stability curve obtained at pH 4.5 is not significantly different from that obtained at pH 7.0. The half-lives of Compound G at the various pH values are all somewhat longer (by 1-2 weeks) than are those for HTH under the same conditions. We believe that two Cl^+ moieties may be expelled rapidly from Compound G as "free chlorine", while the other two Cl^+ moieties may be tightly bound. We plan to perform experiments designed to test whether Compound G still functions as a disinfectant after 50 percent of its total chlorine is lost. It is possible that Compound G may function in a manner similar to the extracellular products of Compound B and HTH.

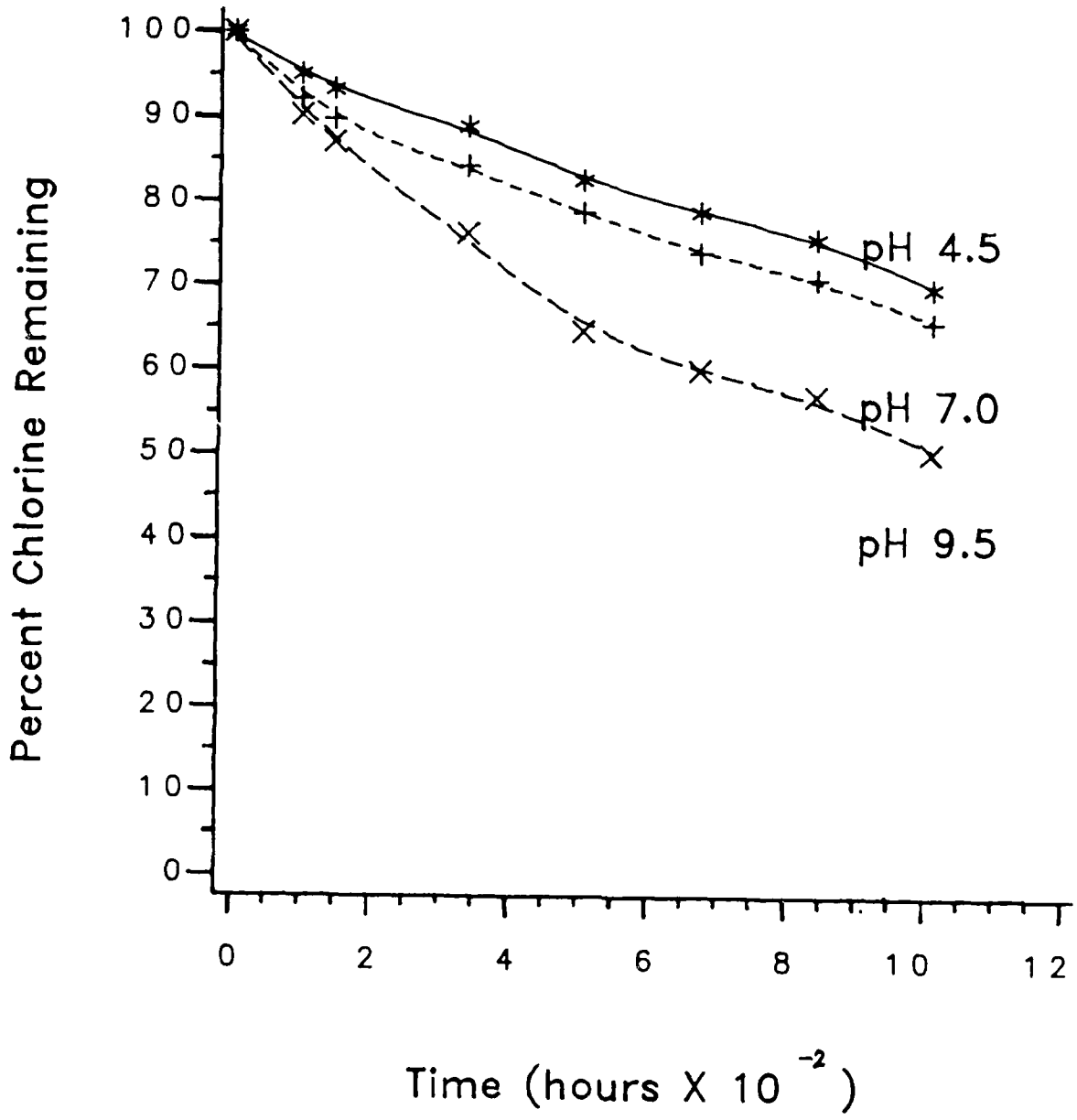


Figure 8. Stability of Compound C in Demand-Free Water at 22°C as a Function of pH.

SECTION VII

TASK II (MECHANISTIC STUDIES)

The goal of Task II is to study the mechanisms of disinfection action of the combined N-chloramine compounds (particularly Compound I) and to compare the findings with those for the free-chlorine compound HTH. During the past year two types of experiments were performed: (1) a comparison of the action of Compound I and HTH on DNA, RNA, and protein syntheses in a microorganism and (2) a comparison of the actions of the two compounds on the activity of an enzyme.

DNA, RNA, AND PROTEIN SYNTHESES

Compound I and HTH solutions containing 1 ppm total Cl^+ were incubated at 37^oC with 2-4 x 10^8 cfu/mL suspensions of Staphylococcus aureus for varying contact times. The solutions containing disinfectant and bacteria were quenched with 0.1 mL of 0.02 N sodium thiosulfate to stop disinfectant action after a given time of contact and were vortexed. Then one microcurie of radioactive label specific for incorporation into DNA tritiated thymidine), RNA (tritiated uridine), or protein (tritiated leucine) syntheses was added to each quenched solution. The bacteria were allowed to contact the radioactive labels for 1 hour in order to allow incorporation of the radioactive precursors into DNA, RNA, or protein. The reactions were terminated by the addition of 1 mL of 20 percent trichloroacetic acid; the latter quickly kills any surviving S. aureus. The bacteria containing the incorporated radioactive DNA, RNA, or protein were collected on glass fiber filters which were washed 5 times with 1 N HCl and twice with ethanol. After drying, the resulting filters were counted by liquid scintillation to determine the efficacies of Compound I and HTH in inhibiting bacterial syntheses of DNA, RNA, and protein.

The results of this series of experiments are shown in Table II. The data in the table indicate that Compound I inhibits DNA synthesis more rapidly than does HTH. However, the reverse is true for RNA and protein synthesis. Thus the mechanisms of action of the two compounds may be quite different. Of course, the fact that HTH does kill S. aureus at lower concentration and shorter contact time than does Compound I may indicate that the destruction of RNA and/or protein syntheses in the organism is more damaging to the organism than is disruption of DNA synthesis.

TABLE 11. PERCENT INHIBITION OF DNA, RNA, AND PROTEIN SYNTHESES IN STAPHYLOCOCCUS AUREUS CAUSED BY COMPOUND I AND HTH

Contact Time (minutes)	Percent Inhibition[a]					
	Compound I			HTH		
	DNA	RNA	Protein	DNA	RNA	Protein
2	60	32	30	23	70	80
5	74	35	60	30	76	92
10	83	50	75	80	90	95
60	100	100	95	100	100	95

[a] The concentration of disinfectants was 1 ppm Cl^+; the concentration of S. aureus was 2×10^8 cfu/mL.

ACTIVITY OF THYMIDYLATE SYNTHASE

Thymidylate synthase catalyzes the reductive methylation of 2′-deoxyuridine-5′-monophosphate (dUMP) by 5,10-methylene-5,6,7,8-tetrahydrofolate to produce thymidylate and 7,8-dihydrofolate. This reaction is the primary de novo source of thymidylate and may be the rate-limiting step in DNA synthesis (35). The enzyme from Lactobacillus casei, which was used in this study, has four sulfhydryl groups, one of which is essential for catalytic activity (36) and is inhibited by many sulfhydryl reagents. Oxidation of a sulfhydryl group by disulfides decreases enzymatic activity, and reduction of the oxidized enzyme with sulfhydryl compounds often completely or partially restores activity (37). Under Task 11 we performed experiments designed to test the effects of Compound I and HTH on the enzyme thymidylate synthase.

Thymidylate synthase was purified from an amethopterin-resistant strain of Lactobacillus casei by the procedure of Lyon and coworkers (38). Purified enzyme preparations had specific activities between 3.0 and 3.5 units/mg when assayed in the presence of 25 mM 2-mercaptoethanol. The enzyme preparations were activated, and 2-mercaptoethanol was removed as described by Aull and Daron (37). Epimeric (±)-H4-folate was prepared by the catalytic hydrogenation of folic acid (39) and was stored at -80°C as a lyophilized powder under argon in sealed serum bottles (40).

Thymidylate synthase activity was determined spectrophotometrically by measuring the increase in absorbance at 340 nm (Gilford Model 250 Spectrophotometer) resulting from the formation of H_2-folate (41). A unit of enzyme activity is defined as that amount catalyzing the formation of 1 micromole of H_2-folate per minute. Protein concentrations were determined from A_{280} measurements and an absorptivity of $1.05 \times 10^5\ M^{-1}cm^{-1}$ for thymidylate synthase (38).

Immediately before use, thymidylate synthase was dethiolated as described by Aull and Daron (37) by applying enzyme that had been activated by dialysis against buffer containing 2-mercaptoethanol to a small column of CM-Sephadex, washing the column with 0.05 M potassium phosphate buffer (pH 6.8) to remove thiols, and eluting the enzyme with 0.05 M potassium phosphate buffer containing 0.5 M KCl. Reaction mixtures were prepared by adding 0.1 mL of a demand-free solution of Compound I or HTH to 0.2 mL of dethiolated enzyme solution and were incubated at 0^{o}C. A control containing 0.1 mL of demand-free water and 0.2 mL of dethiolated enzyme was also prepared for each experiment. Aliquots (usually 20 microliters) were removed from the reaction mixtures and assayed for enzymatic activity. Spectral changes of the enzyme and of tyrosine, tryptophan, and dUMP were monitored using the Gilford Model 250 Spectrophotometer.

The data in Figures 9 and 10 show that Compound I inactivates thymidylate synthase more rapidly and at lower disinfectant concentration than does HTH. However, when dUMP was used to protect the thymidylate synthase (through its active sites), the inactivation of the enzyme due to Compound I was retarded appreciably (Figure 11). The slight protection provided by dUMP against inactivation of the enzyme by HTH is probably due to a direct reaction of dUMP with HTH since the ultraviolet spectrum of a dUMP solution changes significantly upon addition of HTH; no spectral changes of dUMP occur upon addition of Compound I.

No ultraviolet spectral changes were observed when a solution of thymidylate synthase (6.6 μM) was treated with Compound I (final concentration of 0.66 μM), although the enzyme was completely inactivated by this treatment (Figure 12). However, large changes in the enzyme spectrum resulted when enzyme solutions were treated with low concentration (39 μM) of HTH (Figure 12). The absorbance in the region from 265-300 nm gradually decreased over a 1 hour time period, with an isobestic point occurring at about 260 nm suggesting a reaction of HTH with aromatic amino acids. The ultraviolet spectra of solutions of tyrosine and tryptophan were monitored following the addition of Compound I and HTH. Compound I caused no changes in the spectrum of either amino acid, while HTH caused marked decreases in absorbance in the 260-290 nm regions.

It has been shown that cysteine, an amino acid which contains a sulfhydryl group, reacts rapidly with the inorganic compound NH_2Cl (monochloramine) (42). Thus the sulfhydryl groups of cysteine side chains in thymidylate synthase would appear to be likely targets of attack by Compound I. To confirm this, the sulfhydryl groups of thymidylate synthase were blocked with sulfhydryl reagents (p-hydroxymercuribenzoate (PHMP) or methylmethanethiolsulfonate (MMTS)) which inactivated the enzyme before treatment with Compound I or HTH. The control enzyme samples could be completely reactivated by dialysis for 12 hours at 10^{o}C in 50 mM potassium phosphate buffer (pH 8.0) containing 25 mM 2-mercaptoethanol. For protected enzyme treated with Compound I, near complete reactivation could be achieved by incubation for 1 hour at 0^{o}C with 0.15 M 2-mercaptoethanol. In contrast, protected enzyme treated with HTH could not be reactivated at all.

The results of this study show that Compound I is more reactive than is HTH in inactivating thymidylate syntnase. The data also suggest that Compound I reacts more specifically than does HTH with the sulfhydryl groups

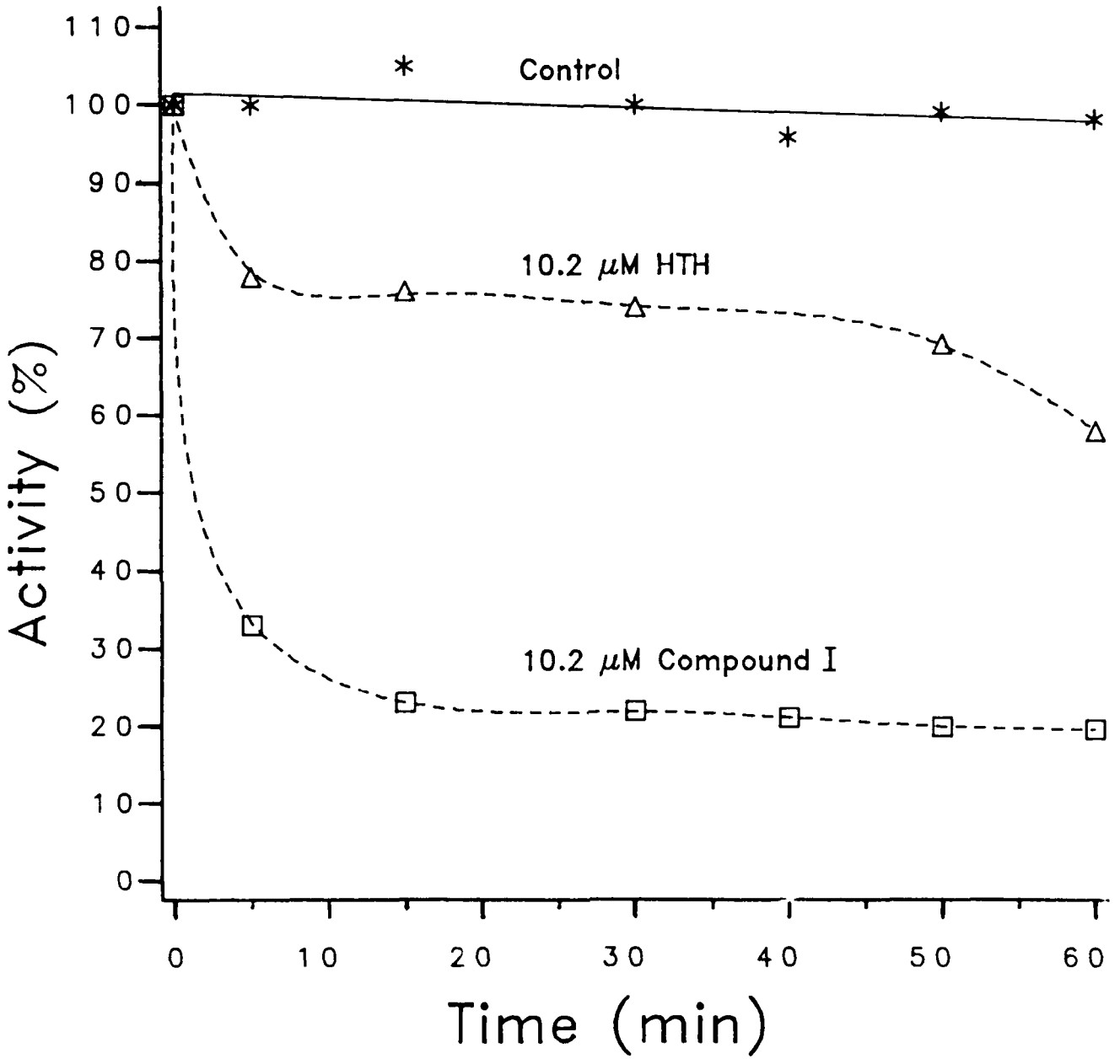
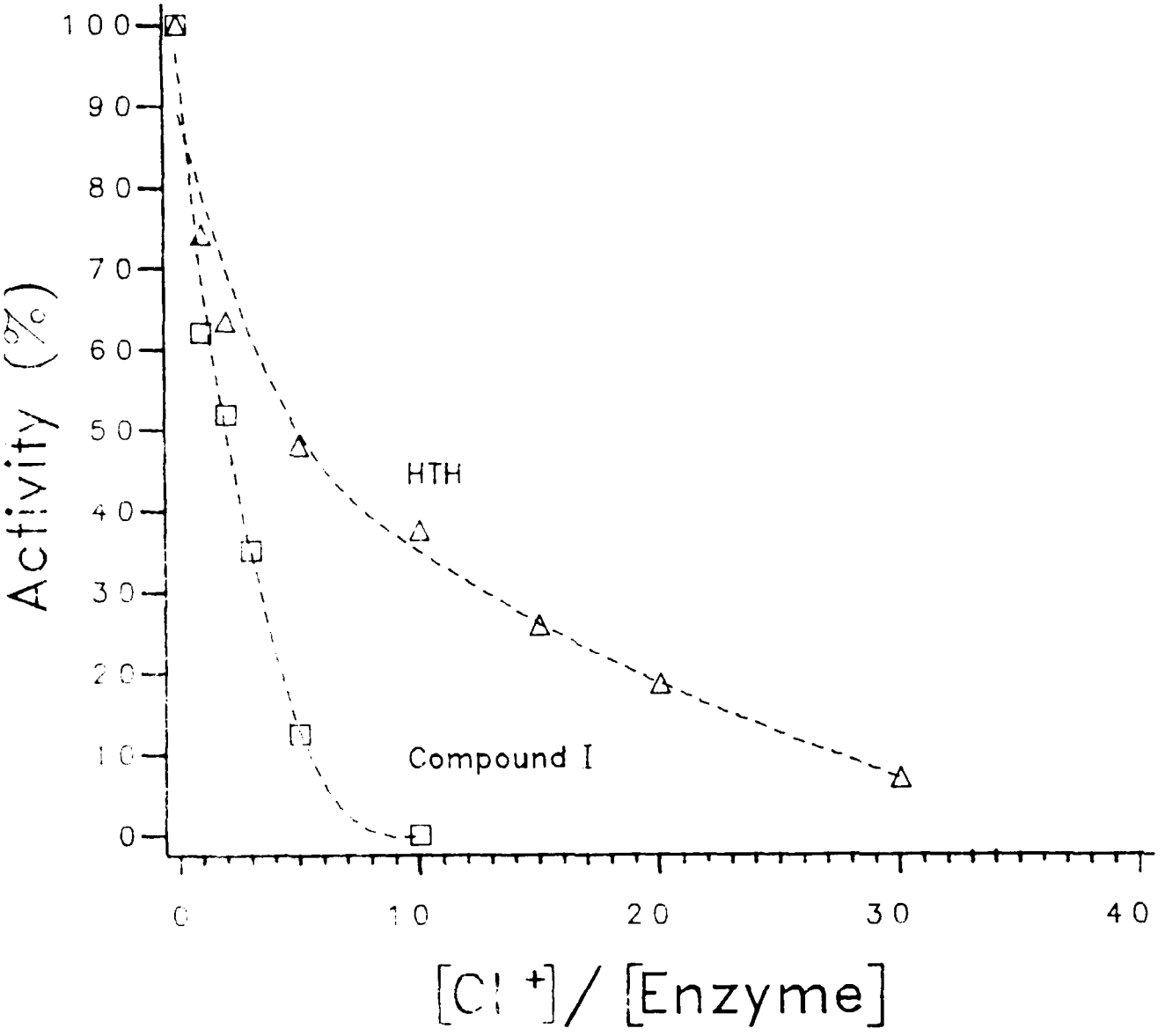


Figure 4. Inactivation of 3.05 μM Thymidylate Synthase by Compound I and HTH.


100
90
80
70
60
50
40
30
20
10
0
Activity (%)
HTH
Compound I
0
10
20
30
40
[Cl+]/ [Enzyme]

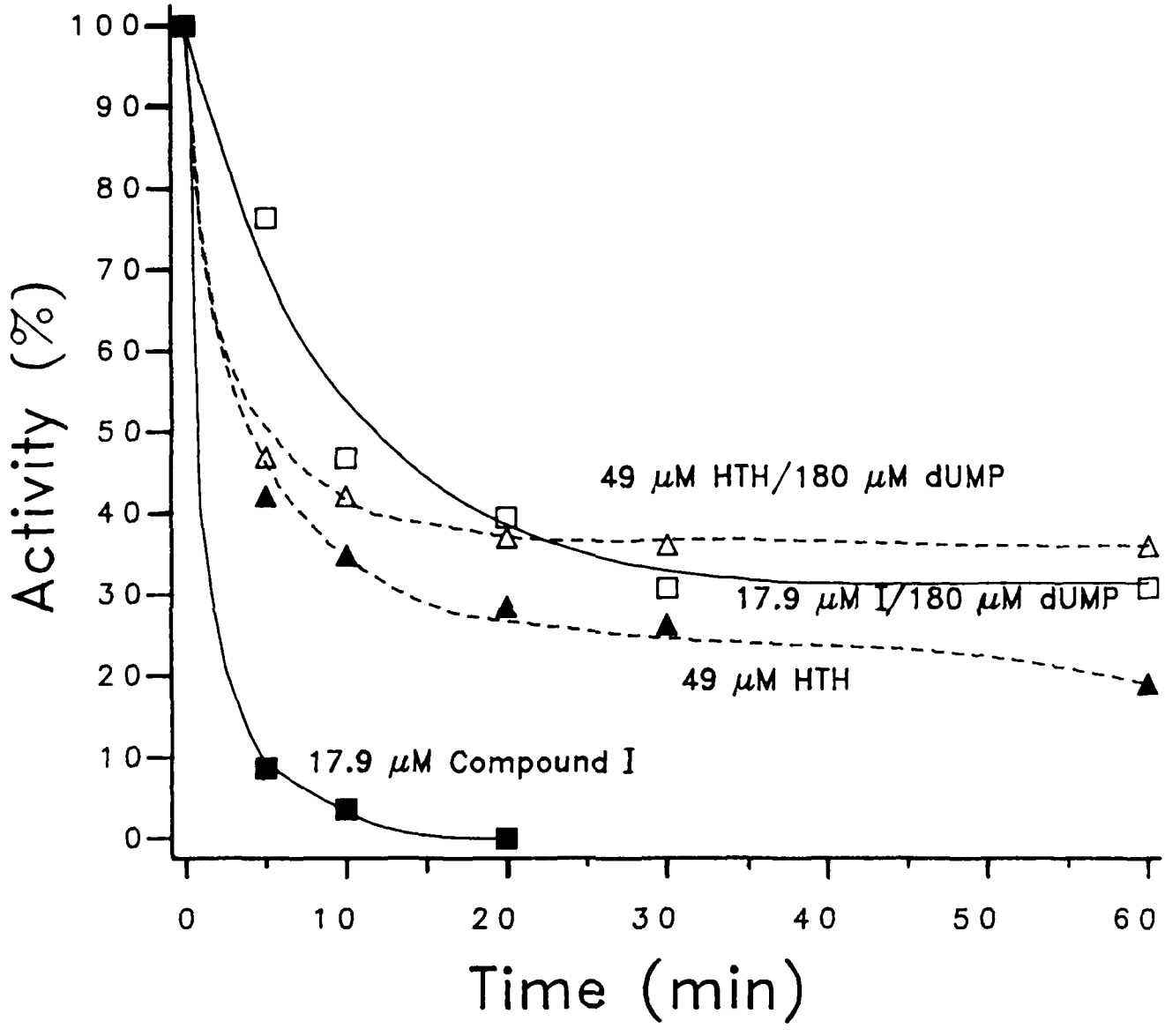
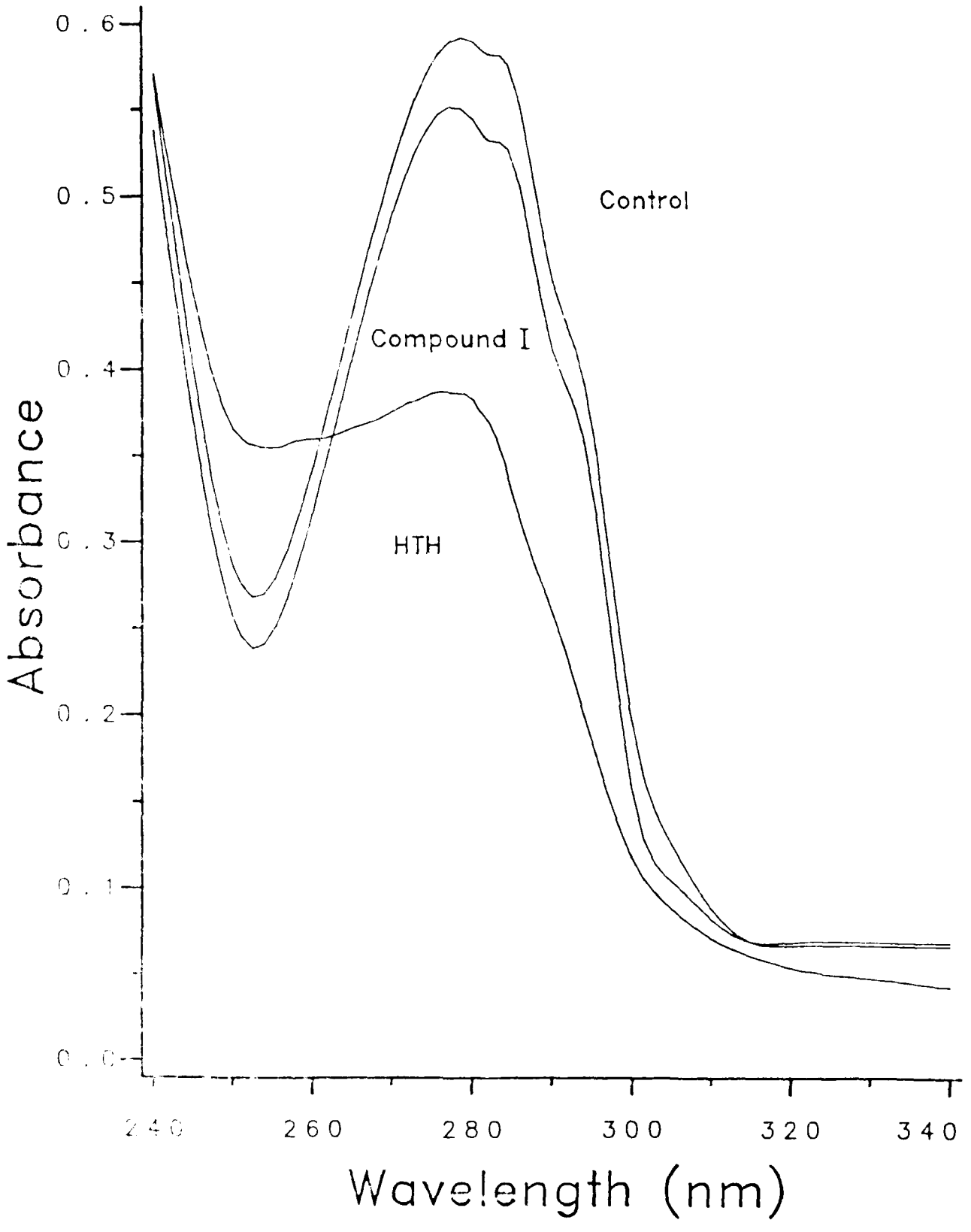


Figure 11. Protection of 2 μM Thymidylate Synthase from Inactivation by dUMP.



[illegible] Ultraviolet Spectra of Solutions of 5.6 μM Benzidolate [illegible] Treated by Compound I (66 μM Cl^-) and by HTH [illegible] for 20 Minutes at 25°C.

of the enzyme and does not react with the aromatic amino acid side chains. Thus the mechanisms of action of Compound I and HTH upon microorganisms may well be quite different. Work here has shown that the order of efficacies of the two disinfectants in killing a broad variety of microorganisms is quite dependent on the nature of the microorganisms (12). Work aimed toward determining whether Compound I is more effective than HTH against organisms containing high concentrations of sulfhydryl groups is in progress.

SECTION VIII

CONCLUSIONS

Mixtures of Compound I and HTH offer considerable promise as military field water disinfectants. The HTH in the mixture provides rapid, initial disinfection through release of "free chlorine", while the Compound I provides long-term disinfection through its stable "combined chlorine". A mixture of 1 part HTH and 10 parts Compound I at a total chlorine concentration of only 1 ppm was able to kill S. aureus initially, and then to continue to kill the organism upon rechallenge at 1 week and again at 1 month. Compound I was found to be considerably more stable than HTH in demand-free water at 37°C at acid or neutral pH conditions. However, at pH 9.5 and 37°C HTH becomes more stable than Compound I in demand-free water, probably because of degradation of the Compound I ring structure catalyzed by strong base at high temperature. Light shielding seems to have little effect on the stability of either Compound I or HTH in demand-free solution. Compound I kills S. aureus more rapidly at 37°C than was the case at 22°C.

Seven N-halamine disinfectants were synthesized during the past contract year. Of these, compounds IB, AB, and C look promising because of their greater bactericidal effect than Compound I; Compound A shows promise because its stability in greater than that of Compound I in water containing organic demands. These compounds will be tested more extensively during the next contract year.

The efficacies of Compound I and HTH against rotavirus have been compared under a variety of conditions. Compound I killed rotavirus at lower concentrations and in shorter contact time than it killed poliovirus type 1. However, HTH was the better disinfectant against both viruses. Continued testing of Compound I and HTH against Entamoeba invadens and Giardia lamblia under all conditions including worst case water has revealed that Compound I kills these protozoa at lower concentration and in shorter contact time than does HTH. The mechanisms of action of the two disinfectants are clearly different and dependent upon the nature of the microorganism under study.

That the mechanistic actions of Compound I and HTH are definitely different is further illustrated by the facts that Compound I tends to disrupt RNA synthesis more effectively than does HTH; the reverse is true for RNA and protein syntheses. Compound I seems to attack sulfhydryl groups on enzymes more rapidly than does HTH. On the other hand, aromatic amino acids react more efficiently with HTH than they do with Compound I.

SECTION IX

RECOMMENDATIONS

The results of the research of the past contract year indicate that mixtures of HTH and Compound I would be better field water disinfectants than is pure HTH. A small amount of HTH would be sufficient for rapid disinfection, with a larger amount of Compound I serving as a long-term disinfectant. A concentration level of 1-2 ppm total Cl^+ should be sufficient for the disinfecting mixture.

Compound I could be used for field disinfection in tropical climates unless the water has high pH (greater than 8.5). Compound I should definitely be used in lieu of HTH for disinfection of field water containing heavy Giardia lamblia contamination.

The research of the past year has produced several N-halamine compounds with desirable disinfection properties. The research should be continued with the goal of synthesis of a new compound which is more germicidal than Compound I, yet which at least approaches Compound I in stability for long-term disinfection action.

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